ARTIGO ORIGINAL
ISSN 1677-5090
DOI: 10.9771/cmbio.v23i2.55152

Discrepancies between red cell phenotyping and genotyping for characterization of the Duffy blood system in blood donors in southern Brazil

Discrepâncias entre a caracterização fenotípica e genotípica do sistema sanguíneo Duffy em doadores de sangue no sul do Brasil

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Abstract

Introduction*: Genetic variants in the ACKR1 (FY) gene, which encode Duffy red blood cell antigens, may result in weakened expression of antigens or null phenotype. Genetic variants are the main reason that led to discrepancies between phenotype and genotype. Molecular methods are a tool for the limitations of hemagglutination assays.* ***Objective:*** *In this study, we investigated discrepant results between serology and molecular tests for the Duffy system in blood donors from a blood center in the northwest region of Rio Grande do Sul, Brazil.* ***Methodology:*** *we studied 80 blood donor samples by serological method, real-time PCR, and gene sequencing.* ***Results:*** *of these 80 samples phenotyped for Fy^a and Fy^b antigens and genotyped for SNPs rs12075 (c.125G>A) and rs2814778 (c.-67T>C), 3 (3.75%) discordant samples were observed. Considering these results, the estimated kappa value for the genotype-phenotype agreement was 0.936 (95% CI 0.865-1.000). We identified an SNP c.265C>T (rs34599082) in one of the samples. We did not identify variants in the other two samples: one of them phenotyped as Fy(a+b-), and the other had phenotype Fy(a-b+). Both were genotyped as FY*A/FY*B genotype, respectively; these samples should express the Fy^b and Fy^a antigens.* ***Conclusion****: genotyping of SNPs rs12075 and rs2814778 were concordant and showed that genotyping and phenotyping are essential to ensure 100% accuracy for Duffy blood group assignments. Sequencing is an important tool for resolving phenotype/genotype conflicts, identifying alleles that carry mutations that lead to weak expression or silencers, and identifying new variants.*

Keywords: *Duffy blood group genotyping; discrepancies phenotype-genotype; serological testing; real-time PCR.*

Resumo

Introdução: as variantes genéticas no gene *ACKR1* (*FY*) que codifica os antígenos do sistema sanguíneo Duffy nas hemácias podem gerar a fraca expressão dos antígenos a fenótipos nulos. As variantes genéticas são as principais causas que levam às discrepâncias entre fenótipo e genótipo. Os métodos de biologia molecular são ferramentas importantes para auxiliarem nas limitações dos testes de hemaglutinação. ***Objetivo:*** foi investigar resultados discrepantes entre sorologia e testes moleculares para o sistema sanguíneo Duffy em doadores de sangue cadastrados em um hemocentro na região noroestes do estado do Rio Grande do Sul, Brasil. ***Metodologia:*** foram analisadas 80 amostras de doadores de sangue pelos métodos sorológicos, PCR em tempo real, e sequenciamento genético. ***Resultados:*** das 80 amostras fenotipadas como Fy^a e Fy^b e genotipadas com os SNPs rs12075 (c.125G>A) e rs2814778 (c.-67T>C), 3 (3,75%) foram discordantes entre os métodos. O valor de *kappa* para a concordância entre genótipo-fenótipo foi de 0,936 (95% CI 0,865-1,000). Foi identificado em uma das amostras o SNP c.265C>T (rs34599082). Não foi possível identificar as variantes nas outras 2 amostras, uma delas com o fenótipo Fy(a+b-) e a outra tendo o fenótipo Fy(a-b+), sendo ambas genotipadas como *FY*A/FY*B*, portanto estas amostras deveriam expressar os antígenos Fy^a e Fy^b. ***Conclusão****:* a genotipagem para os SNPs rs12075 e rs2814778 foi concordante e demonstrou que a genotipagem, assim como a fenotipagem, é essencial para assegurar 100% de acurácia para o reconhecimento dos antígenos do sistema Duffy sanguíneo. O sequenciamento é uma importante ferramenta para resolver os conflitos entre fenótipo e genótipo que surgem quando há presença de alelos que carregam mutações gerando uma expressão fraca ou silenciosa e, também, na identificação de novas variantes.

Palavras-chave: Sistema de grupo sanguíneo Duffy; discrepâncias fenótipo-genótipo; testes sorológicos; PCR em tempo real.

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INTRODUCTION

The Duffy system blood group was first described 70 years ago in a patient multiply transfused[1]. Since its description, a great interest in the field of transfusion medicine, anthropology, evolutionary science, genetics, and malariology has been taken[2]. The Duffy antigen receptor for chemokines (DARC) is a transmembrane glycoprotein found in erythrocytes and other cells non-erythroid cells such as kidney, spleen, and lung epithelial cells[3]. The gene ACKR1 (atypical chemokine receptor 1), also known as FY, the first human gene to be attributed to an autosome, is located on chromosome 1q22-1.q23, is composed of two exons that encode the transmembrane glycoprotein that carries the antigens of the Duffy system[4].

*Duffy system antigens are clinically significant because they can cause severe hemolytic transfusion reactions and hemolytic diseases of the fetus and newborn (HDFN). The main variants, Fy^a and Fy^b antigens, are gene products that differ by a single amino acid (Gly42Asp), encoded by two forms alleles FY*A (FY*01) and FY*B (FY*02), that are differentiated by a single base substitution c.125G>A (rs12075)[5]. The FY*B is the reference allele, and the FY*A allele arises from a 125A>G change on exon 2[6]. This single nucleotide polymorphism (SNP) defines the phenotypes Fy(a+b−), Fy(a−b+), Fy(a+b+) and Fy (a−b−)[7]. Additional gene variants of ACKR1 can cause the weak expression or absence of the Fy antigen, such as the rs2814778, rs34599082, and rs13962 polymorphisms[6]. The weak expression of the Fy^b or Fy^a antigen (Fy^x) can be caused by missense mutations c.265C>T (rs34599082) and/or c.298 G>A (rs13962)[3,6,8]. When associated with FY*B, it is designated FY*02W.01 (Fy^x) and occurs in 1-2.5% of Caucasians, not found in Africans[9]. The c.298G>A variant alone does not result in reduced Fy^b expression[9,10]. In addition, the 145G>T variation, which leads to a change of (Ala49Ser), may also be linked to the weak expression of the Fy^b antigen[9,11]. Although rare, the weak reactivity of the Fy^a antigen has already been described by Lopez et al.[9] (2015). Prior to this study, this had only been found in combination with the FY*B allele.*

*The variation c.−67T>C (rs2814778) in the gene's promoter region leads to non-expression of the Fy^b antigen[12]. The phenotype Fy (a−b−) occurs mainly in Africans, and depending on the region, it has a prevalence of 98-100%[13]. This phenotype occurs due to homozygosity for an FY*B (FY*02N.01) allele with a mutation c.1-67T> C and is also known as erythrocyte silent (Fyb^{es}). This mutation in the FY*B allele causes a truncation of the promoter region and damages GATA-1 erythroid transcription[3,10,13,14]. The c.−67T>C mutation has also been found on the FY*A allele (FY*01N.01) but is rare[9].*

Due to the large number of genetic events that can silence or weaken the expression of antigens encoded by an allele, such as events that occur with Duffy antigens, which can make phenotyping that blood group difficult, leading to specifically dubious serological typing results, genotyping is an essential tool to complement the traditional serological method[15]. The objective of this study was to evaluate the discrepancies between phenotyping and genotyping results for the characterisation of Duffy antigens and to identify ACKR1 gene variants in the hemotherapy of blood donors from service in the region northwest region of Rio Grande do Sul.

METHODOLOGY

Population studied

A total sample of 810 regular repetitive voluntary blood donors of both sexes was collected from the Blood Bank of Santa Rosa (HemoSar), Rio Grande do Sul, Brazil (27°52'16''S 54°28'55''W), between April and July 2019. Of the 810 donors, 10% of the genotyped samples were also phenotyped for quality control. So, 80 were also serologically phenotyped for the Duffy system. Of these 80, all were genotyped and correlated with phenotyping to verify discrepancies[16]. The blood donors agreed to participate through written informed consent. This study was approved by the Ethics Research Committee of the Santa Rosa Health of Foundation (FUMSSAR) (number: 110418) and the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) (CAAE number: 03931018.7.0000.5345).

Serologic Red Blood Cell (RBC) Typing

Duffy phenotypes were determined by hemagglutination using the indirect antiglobulin test (IAT) in gel cards (Bio-Rad, Morat, Switzerland). Following the manufacturer's recommendations, a suspension of red blood cells at 5% was first prepared. For each sample, 500 µl of LISS was transferred to a labelled disposable tube, and five µl of red blood cell pellet of the sample was mixed. Then, the procedure was performed as follows: a microtube labelled with the number of each corresponding sample and the identifier of the serum test used, along with a microtube numbered to match the sample (control sample), was labelled, and the red blood cells were resuspended before use. Next, 50 µl of each red blood cell suspension was transferred into the microtubes, to which 25 µl of serum test were immediately added (except in the microtubes being used as a control sample). These were incubated at 37 °C for 15 minutes and centrifuged for 10 minutes. Finally, we read the reactions according to the interpretation below: agglutinates (on the surface of, or dispersed through the gel) or hemolysis in the microtube constituted a positive result, while a compact red blood cell button at the bottom of the microtube and the absence of hemolysis constituted a negative result. The results were also validated based on the expected results of the positive and negative controls. All samples with phenotyping results discordant from genotyping were double phenotypes.

DNA extraction and Molecular RBC typing

*The genomic DNA was extracted from 5 ml of peripheral blood leukocytes by a standard salting out procedure[17]. The DNA samples were quantified based on optical density at 260 nm (BioSpec-Nano, Shimadzu, Columbia, MD), diluted to 10 ng/µL, and kept at –20 °C for long-term storage. The genotypes of blood group polymorphisms were determined by allele discrimination using a hydrolysis probe with TaqMan 50-nuclease assays on a real-time PCR system (StepOnePlus, Applied Biosystems, Foster City, CA, USA). As listed in Table 1, single nucleotide polymorphisms (SNPs) c.125A > G and c.1-67T>C in ACKR1 (Duffy, rs12075 and rs2814778). The following assays were used: (Thermo Fischer Scientific, Waltham, MA) C___2493442_20 (FY*01/FY*02 − rs12075), C__15769614_10 (FY*02N.01 − rs2814778). The reactions were performed with fast thermal cycling conditions with 1X TaqMan® genotyping master mix, 1X TaqMan® genotyping assay, ten ng of DNA and nuclease-free water (final volume 8 µL) [16].*

Table 1 – *Characterization of the blood group Duffy evaluated in the present study.*

BGS	Gene	allele	dbSNP	Nucleotide	Exon	Amino acid
Duffy	*ACKR1*	FY*01 FY*02	rs12075 rs1299075	c.125A > G	2	*p.Asp42Gly*
Duffy	*ACKR1*	FY*02N.01	rs2814778	c.1-67T > C	promoter	p.0

Blood Group System (BGS)
Data obtained from ISBT (https://www.isbtweb.org)

ACKR1 sequencing

The ACKR1 gene was sequenced in the samples with inconclusive results between phenotype and genotype; sequencing was performed as described in the work by Höher et al.[6] (2020). All the variants identified in the sequencing analysis were confirmed by repeating the PCR reaction and DNA sequencing reactions in both directions (using forward and reverse primers). For the cases of discrepancies, 2488 nucleotides of the ACKR1 gene were sequenced, consisting of analyses of the 1009 bp in two exons, 948 bp of the 5'-untranslated region (UTR), 50 bp of the 3'-UTR, and 481 bp of the adjacent intron. The primer sets for amplification of target exons were designed using the NCBI reference sequence NG_011626.2 through the Primer3 program (http://frodo.wi.mit.edu/primer3/). The amplification reactions were performed in a final volume of 25 µL containing 0.4 mmol/L of dNTPs, 1.5 mmol/L of MgCl2, 0.4 µmol/L of each primer, 1 U of Platinum Taq DNA polymerase, and 100 ng of genomic DNA. Samples were denatured at 94°C for 5min, followed by 35 cycles of 94°C for 30s, 52-56°C for 30s, and 72°C for 30s, with a final extension at 72°C for 5min. The PCR products were purified with 10U Exonuclease I and 2U Shrimp Alkaline Phosphatase (SAP) by incubation for 15 min at 37°C and 30 min at 80°C. The sequencing of the samples was performed using automatic sequencer ABI-PRISM 3130 Genetic Analyzer. After sequencing, the analysis of electropherograms was performed with the Chromas Lite program. ACKR1 blood donors' sequences were compared on MEGA 11[18] and Clustal X v2.1[19] to the consensus sequence NCBI RefSeq NG_011626.2 (range 159,204,013–159,206,500).

RESULTS

Of the total samples of 80 analysed, 8 (10%) donors self-declared as brown, 5 (6.25%) as yellow and 67 (83.25%) as white. In 80 samples phenotyped for Fy^a and Fy^b antigens and genotyped for SNPs rs12075 and rs2814778, only 3 (3.75%) discordant samples were observed (Tables 2 and 3). Considering these results, in relation to the variables analysed, the estimated kappa value for the phenotype-genotype agreement was 0.936 (95% CI 0.865-1.000). In one of the samples was identified the c.265C>T (rs34599082) polymorphism determines the change of p.Arg89Cys.

Table 2 - *Crosstabulation of 80 sample donors between phenotyping and genotyping.*

	Phenotype n (%)				
SNP predicted phenotype n (%)		*Fy (a+b-)*	*Fy (a+b+)*	*Fy (a-b+)*	*All*
	Fy (a+b-)	12 (15.0)	0 (0)	0 (0)	12 (15.0)
	Fy (a+b+)	2* (2.5)	43 (53.7)	1* (1.25)	46 (57.5)
	Fy (a-b+)	0 (0)	0 (0)	22 (27.5)	22 (27.5)

*discordant samples.
Kappa = 0.936 (CI95% 0.857-1.000)

Table 3 - *Genotypes for three donors with discrepancies between phenotype-genotype.*

ID†	Observed phenotype by hemagglutination	SNP predicted phenotype by PCR	Genotype confirmation by Sanger sequencing	Genetic variants		
				rs12075 c.125G>A	rs2814778 c.-67T>C Promoter p.0	Additional variants associated with Fy expression
77	Fy (a+b-)	Fy (a+b+)	*FY*A/FY*B^{w}*	AG	TT	c.265C>T rs34599082
190	Fy (a+b-)	Fy (a+b+)	*FY*A/FY*B*	AG	TT	NI
673	Fy (a-b+)	Fy (a+b+)	*FY*A/FY*B*	AG	TT	NI

W = weak phenotype expression.
NI = not identified
*SNP predicted phenotype from c.125 G > A, c.265C > T and c.−67T > C genetic variants.
† Blood donor sample ID.

*We did not identify variants in the other two samples (ID:190 and 673); one of them phenotyped as Fy(a+b-), and the other had phenotype Fy(a-b+). Both were genotyped as FY*A/FY*B genotype; these samples should express the Fy^{b} and Fy^{a} antigens. There was 100% concordance for all genotypes between the Sanger sequencing and the real-time PCR methodology used to determine the known c.125 G>A and c.−67T>C SNPs. We also researched the literature for the most common variants found in discrepancies between phenotype and genotype for the Duffy system, and these results are described in Table 4.*

DISCUSSION

*When there are genetic variants that can weaken the expression of antigens, for example, c.265C>T (Arg89Cys) and c.298G>A (p.Ala100Thr) variants associated with either the FY*A or FY*B allele, and consequently lead to a reduced expression of the Fy^{a} or Fy^{b} antigens, result in weak or ambiguous antigenic serology reactions. In those cases, it becomes important to apply genotyping of donors and patients9,20–22 in the laboratory routine. Thus, for a reliable application of these tests, the profile of the gene involved in the expression of these antigens must be well elucidated so that the developed assays can identify genetic variations that may be inferring the phenotype[23].*

Any discrepancy should be investigated; false negative results are clinically problematic in blood donors and recipients as they can induce alloimmunisation and transfusion reactions[20]. Resolving phenotypic and genotypic discrepancies, in addition to providing more similar and safer blood units, can also contribute to the identification of new allelic variants and blood group antigens not yet described[10,20,24].

In this study, we performed a screening of genetic variants by direct sequencing of the ACKR1 gene and its proximal promoter region in blood donors with phenotype-genotype discrepancies. After analysing the agreement between the serological and molecular techniques for the variables rs12075 and rs2814778 and after identifying the rs34599082, the Kappa coefficient was calculated, and the value was above 0.957 (95% CI: 0.897 -1.000), showing high agreement between the techniques.

*We identify one variant distributed throughout the whole gene: the missense variant (c.265C>T, rs34599082) in a heterozygous (FY*A/FY*B^{w}) state in 1 (ID 77) donor. This variant, when associated with FY*B, can lead to weak expression of the Fy^{b} antigen (Fy^{x,w}). Phenotypically, the Fy^{w} antigen can only be detected using potent anti-Fy^{b}, but unfortunately, reagents capable of detecting Fy^{w} were not available[25]. In these situations, genotyping definitively resolves these dubious cases.*

*The SNP c.298G>A (rs13962) by itself has not been associated with altered antigen expression Fy^{b} [26]. Differently, when associated with the SNP c.265C>T SNP, it leads to weak expression of the Fy^{b} [9,26,27]. There were no changes in Fy^{a} antigen reactivity. Some works[9,26,27] have suggested that the polypeptides generated by the FY*A allele are inserted into the cell membrane more efficiently than those produced by the FY*B allele. Although the Fy^{b} antigen is down-expressed in erythroid cells, it is normally expressed in other tissues. Therefore, there will be no production of anti-Fy^{b} antibodies. In these cases, they may receive Fy^{b} RBCs. Thus, the use of serology and genotyping would be useful to reduce the need for a Fy(b-) phenotype present in only one-third of Brazilians, facilitating the search for a compatible unit and accelerating the transfusion process[25].*

For c.125 G>A and c.−67T>C SNPs, we had 100 % concordance for all genotypes between the Sanger sequencing and the real-time PCR. After analysing these variants, our discrepancy was around 3.75% (3/80), and when identifying the variant c.265C>T SNP, this discrepancy stayed at 2.5% (2/80). Our results are similar to the results reported by Lopez et al.[22] (2015) and Höher et al.[6] (2020), which had a discrepancy of 3.22% (5/155) and 2.87% (11/382) after analysing c.125 G>A and c.−67T>C SNPs. After including the analysis of c.265C>T and c.298G>A SNPs, the discrepancy was 1.93% (3/155) and 2.6% (9/382), respectively (see Table 4). Overall, comparing the other work, the average discrepancy was 3% when analysed for SNPs rs3499082 and/or rs13962, it was 1.97%. Thus, an appropriate correlation between phenotype and genotype requires a complete analysis of the c.125 G>A, c.−67T>C and c.265C>T, c.298G>A SNPs.

Table 4 - *Comparison between the present study and others in the discrepancies found for the Duffy system.*

Reference	Total sample	Geno-feno discrepancies for Duffy after genotyping rs12075 and rs2814778 n (%)	Molecular cause identified	Cause	Discrepancy due to variants c.265C>T (rs3499082) and/or c.298G>A (rs13962) FY*01W.02 e/ou FY*02W.01 n (%)	Discrepancies for other variants in the ACKR1 or gene with no identified cause	Cause	Molecular testing
Present study	80	3 (3.75)	1	c.265C>T	1 (1.25)	2 (2.5)	NI	Real-time PCR and Sequencing
Guelsin et al.,[28] 2010	38	2 (5.26)	1	c.265C>T	2 (5.26)	0		PCR-RFLP and BeadChip DNA
Lopez et al.,[22] 2015	155	5 (3.22)	4	c.265C>T c.719delG c.287G>A c.281-295del	2[a] (1.29)	3 (1.93)[a]	c.287G>A c.719delG c.281--295del	Real-time PCR and Sequencing
Łukasik et al.,[29] 2019	494	17 (3.44)	2	c.298G>A c.265C>T	17 (3.44)	0		Real-Time PCR and Sequencing
Pellegrino Junior et al.,[25] 2001	216	3 (1.38)	2	c.265C>T c.298G>A	3 (1.38)	0		PCR-RFLP
Höher et al.,[6] 2020	382	11 (2.87)	9	c.298 G>A c.−627C>T c.−541C>T c.−284C>T c.−943delC c.21+115T>C c.21+150C>T c.21+235delT c.22−58A>G	2[b] (0.52)	9 (2.61)[b]	c.−541C>T c.22−58A>G c.21 + 150C>T c.21 + 235delT c.21 + 115 >C c.−284C>T c.−943delC c.−627C>T	Real-time PCR and Sequencing
Total	1365	41			27	14		
Mean (%)		**3.00**			**1.97**	**1.03**		

[a] One sample with the SNP c.287G>A and c.287G>A and another with c.719delG and c.265C>T
[b] Six samples with the variant c.21 + 150C>T, two also had the variant c.22−58A>G and one the c.298 G>A.
NI = not identified

In samples where variants were not determined, these variants could possibly be in an unexplored region of the ACKR1 gene or even other genomic regions and could lead to possible effects on the expression and structure of DARC, as some studies have reported [6]. Analysis of the conserved noncoding region has shown that noncoding DNA may also enrolled in biological functions [30]. These non-coding regions can have various regulatory functions within the genome, such as interacting with transcription factors and miRNAs, creating splice sites and acting as exonic splicing enhancers [6,31]. Functional analysis of non-coding SNPs report mutations in splice sites and promoter regions as underlying mechanisms in altered expression of blood group phenotypes[6,32].

CONCLUSION

As genotyping proceeds, it is important to know the range of FY alleles polymorphisms when predicting the phenotype of antigens. Another important issue is that not every complex case that presents a discordant phenotype-genotype will be limited to null alleles. In this case, sequencing can be used to overcome problems and supplement SNP-based genotyping. In summary, where 100% accuracy is required, genotyping in conjunction with serology is essential, while for more complex cases, sequencing is a valuable complement to resolve discrepancies.

FUNDING

This work was supported by a grant from the Coordination for the Improvement of Higher Education Personnel - CAPES, Brazil.

ACKNOWLEDGEMENTS

S.S. and D.D. performed the research and wrote the first draft of the manuscript; M.F. and S.A. designed the research study; S.A. contributed essential reagents or tools; T.B. and S.S. acquired and analysed the data; M.F. supervised the research and reviewed and edited the manuscript.

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Submetido em: 02/12/2023
Aceito em: 29/07/2024