SMIM1 polymorphisms in a donor population from southeast Brazil and their correlation with VEL expression

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Background. Vel is a high frequency blood group antigen and its alloantibody is involved in haemolytic transfusion reactions. After elucidation of the molecular basis of the Vel-negative phenotype defined by a 17-base pair deletion in *SMIM1*, genotyping has been the technique of choice to identify the Vel-negative phenotype, and molecular investigations have contributed to explain Vel expression variability. The present study was aimed at screening for Vel negative blood donors and characterising the genetic changes found in Brazilian donors with altered Vel expression.

Materials and methods. Molecular screening for the S*MIM1*64_80del* allele was performed in 1,595 blood donor samples using a SNaPshot protocol previously standardised in our laboratory. Four hundred donor samples were also submitted to serological screening using a polyclonal anti-Vel from our inventory. Samples with variability in antigen strength were selected for *SMIM1* sequencing.

Results. No homozygous S*MIM1*64_80del* allele was found and the S*MIM1*64_80del* allele frequency was 1.01%. Different patterns of reactivity were observed in serological testing varying from negative to 3+. Through sequencing analysis we highlighted two polymorphisms: rs1175550 and rs6673829. The minor G allele of rs1175550 was found in 16/20 samples reacting 3+, while the major A allele was found in 21/23 samples reacting 2+. Regarding rs6673829, the minor A allele was present in 14/23 and 3/20 samples reacting 2+ and 3+ respectively.

Discussion. We included molecular VEL screening in a previously standardised SNaPshot protocol, which besides enabling detection of Vel-negative donors, also searches for eight other rare blood types. Additionally, the present study demonstrated that although the S*MIM1*64_80del* allele is responsible for some variation of Vel phenotype in this donor population, Vel expression is also controlled by molecular changes in *SMIM1* intron 2.

Keywords: Vel expression, SMIM1, Vel genotyping, rs1175550, rs6673829.

Introduction

Vel antigen was first described in 1952 by Sussman and Miller[1] after the identification of an antibody to a new high prevalence antigen in a patient who suffered an acute intravascular transfusion reaction[1]. However, it was not until 2013 that the genetic basis of Vel was determined, by three independent groups[2-4], who showed that Vel antigen is carried by a single-pass membrane protein, known as small integral membrane protein 1 (SMIM1). The corresponding gene (*SMIM1*; NM_001163724.2) is composed of four exons and its ATG initiation codon is located in exon 3, producing a 78-amino acid protein. The Vel-negative phenotype is caused by a 17-bp deletion in *SMIM1* (c.64_80del), which completely abolishes SMIM1 protein expression.

Considering the clinical significance of anti-Vel, which is responsible for acute or delayed severe haemolytic transfusion reactions, the identification of Vel-negative donors is important to supply safe blood transfusions for alloimmunised patients. However, the Vel-negative phenotype is considered rare worldwide. Studies in Europe showed a frequency of 0.025%[5], similar to the frequency recently determined in a Brazilian population (0.021%)[6]. The number of Vel-negative donors is also limited by screening difficulties, since there are no serological reagents commercially available. Phenotyping is further complicated by large individual variation in Vel expression and by the instability of the human polyclonal antibodies used for the phenotyping.

After elucidation of the genetic basis of Vel, high throughput molecular screening has come to be the best choice for finding Vel-negative donors. Molecular tools are also required to explain altered expression of Vel antigen in some samples. Negative or weak reactions are generally related to a 17-bp deletion in *SMIM1* in a heterozygous state[2-4], although other nucleotide changes have been described to be involved in the regulation of

Vel expression, such as the missense mutation 152T>A or 152T>G[3]. The major A allele of the single nucleotide polymorphism (SNP) rs1175550, located in intron 2 of *SMIM1*, a regulatory region in erythroblasts, is associated with decreased *SMIM1* transcript levels[3,7].

In this context, the present study aimed to establish a strategy of molecular screening to search for Vel-negative blood donors and to characterise the genetic changes present in Brazilian donors with variable Vel expression.

Materials and methods

Samples

A total of 1,995 blood donor samples from Colsan (Blood Collection Centre), São Paulo, Brazil, were included in this study: 1,595 were submitted to molecular screening and 400 were submitted to serological screening. Samples showing negative results in serological testing and those with variability in the strength of Vel (3+ or weaker) were selected for *SMIM1* sequencing. All donors agreed to participate in this study and signed informed consent.

Serological screening

Vel typing was performed using a polyclonal anti-Vel in an indirect antiglobulin test in gel (Grifols Movaco S.A., Parets del Vallès, Spain), adding 1% red blood cell suspension to the low ionic strength solution/Coombs card containing 25µL of undiluted anti-Vel reagent. After incubation and centrifugation, the reactivity was graded as negative, weak (w), 1+, 2+, 3+ or 4+.

Molecular screening

Genomic DNA was extracted from the buffy coat of peripheral blood with a QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The DNA concentration and purity were calculated by measuring the optical density at 260 and 280 nm using a NanoDrop 2000 Spectrophotometer (Thermal Scientific, Uniscience Inc., São Paulo, SP, Brazil) and DNA samples were kept at –20 °C.

VEL genotyping was included in our SNaPshot protocol previously standardised for the identification of rare alleles[8]. Although the molecular mechanism is a deletion rather than a SNP, the inclusion of *VEL* genotyping in our SNaPshot protocol was possible because the internal primer was designed upstream of the deletion and the subsequent nucleotide differs if the deletion is present, as previously reported[9]. Primers were designed using the Primer 3 programme (http://frodo.wi.mit.edu/): forward 5'ACCCTGGGCAAATGACTCTAC 3', reverse 5'GTGAGAACCAACTGGAGGTGA 3' and internal primer 5'CGGCAGCAGGGACGGAGTC 3'. A polyA tail containing 56 adenines was added to the internal primer, allowing fragment analysis without interfering with the other alleles. Fragments were analysed in a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and interpreted using GeneMapper Software v4.1 from Applied Biosystems (Figure 1). To validate the assay, we used 1,000 samples genotyped in parallel with the previously described polymerase chain reaction (PCR) - restriction fragment length polymorphism (RFLP) protocol[4].

DNA sequencing

From 400 samples submitted to serological screening, sequencing was performed in 51 samples which presented different patterns of reaction. *SMIM1* exons and introns were amplified using the primers listed in Table I. PCR was performed with 50-100 ng of DNA, 0.25 µM of each primer, 0.25 mM of each dNTP, 2.5 mM of $MgCl_2$, 1.0 U Taq DNA polymerase and Taq buffer 1x. PCR amplification was performed in a Veriti™ 96-well Thermal Cycler (Applied Biosystems) using the following conditions: denaturation at 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 sec, annealing temperature (described in Table I) for 30 sec and 72 °C for 60 sec, and a final extension step of 72 °C for 10 min. After purification with 0.5 µL of Exonuclease I and 1.0 µL of Shrimp Alkaline Phosphatase (Fermentas, Hanover, MD, USA) the PCR products were submitted to a sequencing reaction using 0.15 µM of forward or reverse primer and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Products were purified using the BigDye X-terminator Kit (Applied Biosystems), sequenced in a 3500xL Genetic Analyzer (Applied Biosystems) and analysed using CLC Sequence Viewer v7.6 software (QIAGEN, Aarhus, Denmark).

Statistical analysis

Allele frequencies among samples with distinct antigen strengths were compared using Fisher's exact test, considering p-values <0.05 as statistically significant. The data were processed in GraphPad Prism software, version 5.04 (GraphPad Software, San Diego CA, USA)

Results

Molecular screening for the Vel-negative phenotype

We successfully included *VEL* genotyping in our previous SNaPshot protocol designed to screen for rare donors[8] (Figure 1). In the validation process we obtained 100% agreement with PCR-RFLP and the genotyping of other alleles was not impaired. Among the 1,595 blood samples submitted to molecular screening, 32 (2.0%)

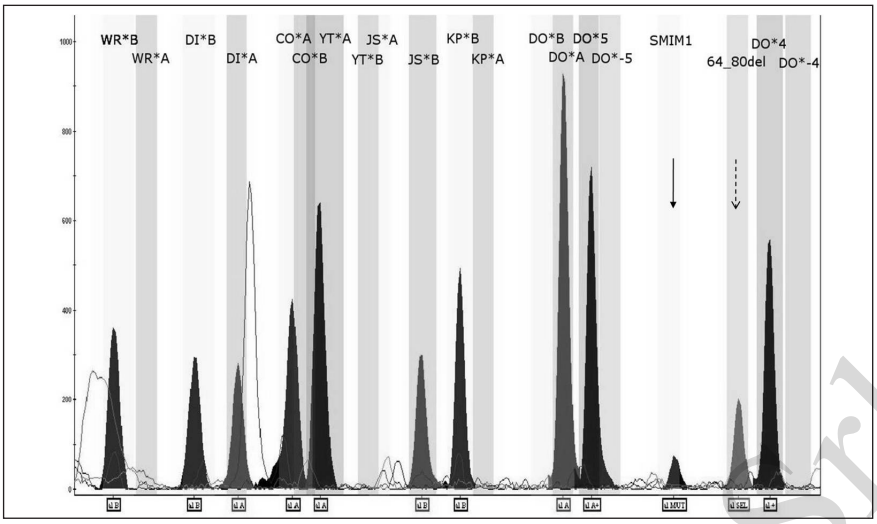

Figure 1 - Gene Mapper electropherogram of representative SNaPshot fragment analysis of a sample heterozygous for the *SMIM1*64_80del* allele.
Bins are labelled at the exact place where each allele should migrate. The X axis represents the fragment size (nucleotides), the Y axis represents relative fluorescence units (rfu). The peak related to wild-type *SMIM1* is indicated by a solid arrow, while the dashed arrow represents the *SMIM1*64_80del* allele.

Table I - Oligonucleotides used for SMIM1 sequencing.

Primer name	Amplified region	Primer sequence	Product size	Annealing temperature
SMIM1_1 F SMIM1_1 R	Promoter region	ggaagggactctccccatta aaggtctggggccagtag	571pb	60 ºC
SMIM1_2 F SMIM1_2 R	Exon 1 - Exon2	Ttccggaccagacacctc caggaaggccctaggtacac	585 pb	60 ºC
SMIM1_3 F SMIM1_3 R	Exon 2 - Intron 2	Ttagcaagatcggcttctcc agccataaagtgtgccttcg	967 pb	60 ºC
SMIM1_4 F SMIM1_4 R	Intron 2	Ctgggaccagaatgggatt gagacaggagggtcgtaggg	837 pb	60 ºC
SMIM1_5 F SMIM1_5 R	Intron 2 -Exon 3	gcaaagatgagtcccaggtc gagacaccagcctgctatgg	994 pb	60 ºC
SMIM1_6 F SMIM1_6 R	Exon 3 - Exon 4	Cccttagtgcccctctccta gtgcccagctgtttgtaggt	941 pb	60 ºC
SMIM1_7 F SMIM1_7 R	3'UTR	TTCATCCTGGGCTACCTCAC tccaccgttcattcaacaga	832 pb	60 ºC
SMIM1_I2a-F[a] SMIM1_I2b-R	Intron 2	Acccacacaggctgaggacac gggtctgtcgcctgacttaaca	452 pb	64 ºC

Primers used to sequence the *SMIM1* gene of the samples with different reactivity patterns. [a]Pair of primers described by Cveji *et al*.[3]

samples were heterozygous for the 17 bp deletion in *SMIM1* and no sample was homozygous. The frequency of S*MIM1*64_80del* allele in this blood donor cohort from the Southeast Brazil was, therefore, 1.01%.

rs1175550 and rs6673829 associate with Vel antigen expression

The pattern of reactivity among the 400 samples serologically tested varied from negative to 3+. Four samples gave negative results and 47 samples showed a substantial individual variation in antigen strength, ranging from weak (less than 1+) to 3+. In these 51 samples, the *SMIM1* gene was sequenced completely and several nucleotide changes were detected, mainly in introns, although they were not uniform among the samples with the same pattern of reactivity (*data not shown*). The polymorphism c.152T>A and c.152T>G, previously described to be implicated in decreased Vel expression[7], was also investigated but no sample had this polymorphism. Similarly, no changes in the 3'UTR of

SMIM1 were observed in investigated samples. Although the 58G>A change in the UTR is not strongly associated with Vel expression, it could not be excluded that this nucleotide change has an influence[7].

All samples with a negative result and with very weak reactivity in serological testing were heterozygous for the 17-bp deletion in *SMIM1*. In contrast, one sample with the same molecular background showed a haemagglutination reaction of 2+.

The variability in Vel expression was correlated with specific polymorphisms in *SMIM1* intron 2. In our study, the nucleotide change rs1175550 was strongly associated with Vel antigen expression, since 16 out of 20 samples with a haemagglutination reaction of 3+ had the minor G allele and 21 out of 23 samples with a 2+ reaction had the major A allele ($p<0.0001$). Our results suggest that the SNP rs6673829 may also interfere with the expression of Vel, since the minor A allele was found in 14 of 23 (60.97%) and in 3 of 20 (15.0%) samples with a reactivity of 2+ and 3+, respectively. The distribution of the allele frequency shows a statistically significant difference between the samples with a reactivity of 2+ and 3+ ($p=0.039$). The molecular findings are described in Table II and plotted in Figure 2.

Discussion

Considering the difficulty in finding Vel-negative blood donors and the scarcity of studies in the Brazilian population, the present study established the S*MIM1*64_80del* allele frequency in a cohort of blood donors from southeast Brazil using a SNaPshot protocol. In addition, variations in Vel expression were evaluated and correlated with polymorphisms in *SMIM1*. Although we sequenced the entire *SMIM1* gene, we found polymorphisms related to serological results only in intron 2, a region that is responsible for gene transcription. Our results strengthen the effect of

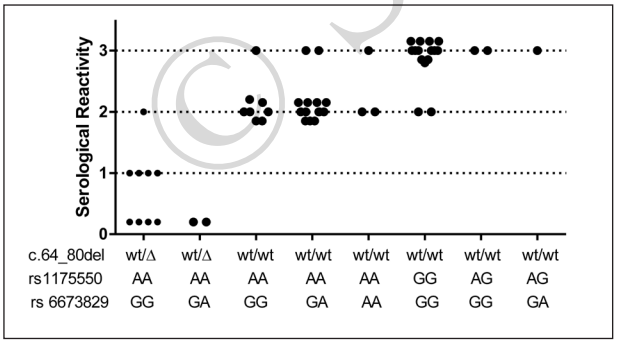

Figure 2 - Correlation between serological reactions and *SMIM1* genotypes.
Vel expression was higher (3+) in donors homozygous for the minor G allele of rs1175550 and for the major G allele of rs6673829. Most samples showing 2+ reactivity had genotypes AA rs1175550 and GA rs6673829. wt: wild type; Δ: deletion.

Table II - *SMIM1* genetic profile of samples with different patterns of reactivity.

ID	Reactivity	rs1175550	rs6673829	c. 64_80del
		Minor allele		
		G	A	Δ
5	0	AA	GA	wt/Δ
6	0	AA	GG	wt/Δ
7	0	AA	GA	wt/Δ
8	0	AA	GG	wt/Δ
71	w	AA	GG	wt/Δ
3	w	AA	GG	wt/Δ
11	w	AA	GG	wt/Δ
12	w	AA	GG	wt/Δ
13	2+	AA	GG	wt/Δ
1	2+	AA	GA	wt/wt
2	2+	AA	AA	wt/wt
4	2+	AA	AA	wt/wt
9	2+	AA	GA	wt/wt
10	2+	AA	GG	wt/wt
14	2+	AA	GA	wt/wt
15	2+	AA	GA	wt/wt
606	2+	AA	GA	wt/wt
16	2+	AA	GA	wt/wt
48	2+	AA	GA	wt/wt
51	2+	AA	GA	wt/wt
69	2+	AA	GG	wt/wt
49	2+	AA	GA	wt/wt
22	2+	AA	GA	wt/wt
80	2+	GG	GG	wt/wt
79	2+	AA	GG	wt/wt
68	2+	AA	GG	wt/wt
92	2+	AA	GA	wt/wt
84	2+	AA	GA	wt/wt
90	2+	AA	GG	wt/wt
73	2+	AA	GG	wt/wt
78	2+	GG	GG	wt/wt
23	3+	GG	GG	wt/wt
24	3+	GG	GG	wt/wt
53	3+	GG	GG	wt/wt
50	3+	GG	GG	wt/wt
67	3+	AA	AA	wt/wt
20	3+	AG	GA	wt/wt
75	3+	GG	GG	wt/wt
77	3+	GG	GG	wt/wt
76	3+	AG	GG	wt/wt
85	3+	AA	GG	wt/wt
97	3+	AA	GA	wt/wt
91	3+	GG	GG	wt/wt
96	3+	GG	GG	wt/wt
74	3+	AG	GG	wt/wt
94	3+	AA	GA	wt/wt
6	3+	GG	GG	wt/wt
11	3+	GG	GG	wt/wt
20	3+	GG	GG	wt/wt
26	3+	GG	GG	wt/wt
24	3+	GG	GG	wt/wt

w=weaker than 1+; wt: wild type; Δ: deletion.

the SNP rs1175550 and 17-bp deletion in heterozygous state in the variation of Vel expression, and suggest that the minor A allele of rs6673829 may also be related to the reduction of Vel antigen expression.

Identification of Vel-negative donors is crucial for blood transfusion support to Vel negative patients, but it is a great challenge in immunohematology centres. Until the identification of the molecular basis of Vel, the screening for Vel-negative blood donors was performed exclusively with human antisera in a labour-intensive process that could generate misinterpretation. Once the molecular basis had been determined, some molecular protocols were proposed to identify and screen Vel-negative individuals, such as PCR-RFLP, high resolution melting, single-strand polymorphism-PCR and real-time PCR[4,6,10-12]. However, with regards to screening for rare blood donors, the protocol proposed here has the advantage of genotyping nine types of rare blood simultaneously. We, therefore, developed a strategy to screen for Vel-negative blood donors by including the genotyping of S*MIM1*64_80del* allele in a SNaPshot protocol which had been previously standardised in our institution to screen for rare donors[8]. No Vel-negative donor was identified from 1,595 samples studied. The frequency of the S*MIM1*64_80del* allele in this donor population was 1.01%, differing from that in a previous study performed in blood donors from the southeast in whom the reported frequency was 0.22%[6]; on the other hand, it was similar to the frequency found in a study performed in donors from the south of Brazil (1.12%)[11]. As the Brazilian population is considered admixed, differences in the allele frequency may occur, especially in the southeast, a region that has received immigrants from many countries. Comparing the frequency around the world, we observed that the frequency of the S*MIM1*64_80del* allele in our study is closer to that in the European population, in which it varies from 1.46% to 2.9%[2,3,7,10], while differing from that found in African and Asian individuals in whom allele frequencies of 0.56 and 0.6%, respectively, have been reported[7].

The variability of Vel expression observed in our study was previously reported by other authors[3,7,10]. In most of our samples, agglutination reactivity varied from 2+ to 3+, and was strongly associated with molecular changes in *SMIM1* intron 2. As previously shown, the minor G allele of rs1175550 leads to increased Vel expression, since this base provides low binding affinity to repressive factors[3]. On the other hand, the major A allele is related to a reduced expression of Vel antigen. Our results confirmed this effect of rs1175550, since 91% of the samples with 2+ reactivity had the major A allele, whereas 80% of the samples with 3+ reactivity carried the G allele. In addition, the rs6673829 shows a statistically different distribution among the samples with variations in Vel expression, with the minor A allele present in 60% of the samples with 2+ reactivity and in 20% of the samples with a 3+ agglutination reaction. Further studies are, however, necessary to confirm the role of rs6673829 in Vel expression.

Excluding the samples in which Vel expression could be explained by the presence of rs1175550 and rs6673829 SNP in *SMIM1* intron 2, two samples with a reactivity of 2+ and one sample with a reactivity of 3+ were in disagreement with our findings. Haer-Wigman *et al.*[7] showed that the *SMIM1*152A* allele is involved in the reduction of Vel expression, since the encoded amino acid is very close to the transmembrane region in the SMIM1 protein. However, in our study no changes were observed in either this nucleotide position or in the 3'UTR, where the c.58G>A mutation might reduce Vel expression. Thus, in these three samples the variation in Vel expression may be influenced by genetic factors outside *SMIM1* or by environmental factors, as already suggested by Haer-Wigman *et al*[7].

In our study, Vel expression varied from 0 to 2+ even in individuals with both the *SMIM1*64_80del* allele and the major A allele of rs1175550. In contrast, other studies showed that individuals heterozygous for the deletion but carrying the major A allele of rs1175550 have Vel-negative or weak phenotypes, while, the minor G allele on the non-deletion haplotype can provide normal Vel expression[6,7,10,11]. Additionally, two samples typed as Vel-negative carried the minor A allele of rs6673829, which could be an additional factor related to a reduction in Vel expression, although the other two "Vel-negative" samples had a guanine in the SNP rs6673829.

Conclusions

In conclusion, we introduced a strategy to identify Vel-negative blood donors based on the detection of a deletion in the *SMIM1* gene in a SNaPshot protocol used to screen for high prevalence negative antigens in order to supply safe transfusion to patients with rare blood types. We also showed that in our donor population, Vel expression is controlled by molecular changes in *SMIM1* Intron 2 region, mainly the SNP rs1175550, although other polymorphisms can be involved. Our data reinforce the concept that molecular screening can be considered the gold standard method to predict the Vel phenotype. Knowledge of the molecular background associated with the variations in Vel expression in different populations can be helpful to design reliable methods to screen for Vel-negative blood donors.

Authorship contributions

CPA performed molecular tests, analysed the results

and wrote the paper. JGM and TAdPV were responsible for the serological tests. DG helped with the molecular tests. RDdMP selected the samples. FRML and AJPC contributed to writing the paper. LC contributed to the study design and reviewed the data and the paper. All Authors read and approved the final manuscript.

The Authors declare no conflicts of interest.

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