Molecular Basis of KELnull Phenotype in Brazilians

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Summary

Background: KELnull (K_0) persons can produce clinically significant anti-KEL5 antibody after transfusion and/or pregnancy, requiring K_0 blood transfusion when indicated. 37 K_0 alleles have been reported in studies over different populations, but none in Amerindian-Caucasian descendants from South America. The aim of this study was to identify the molecular basis of K_0 phenotype in Brazilians. **Methods:** We investigated three K_0 samples from different Brazilian blood banks (Recife, Manaus, and Vila Velha) in women with anti-KEL5. KEL antigen typing was performed by serologic techniques, and the K_0 status was confirmed by flow cytometry. PCR-RFLP and DNA sequencing of the *KEL* coding and exon-intron regions were also performed. **Results:** RBCs of the 3 patients were phenotyped as KEL:-1,-2,-3,-4,-7. The 3 patients had the same *KEL**02/02 genotype and were negative for *KEL**02.03 and *KEL**02.06 alleles. The Recife K_0 patient was homozygous for IVS16 + 1g>a mutation (*KEL**02N.31 allele). The flow cytometry with anti-KEL1, anti-KEL2, anti-KEL3, anti-KEL4, and anti-CD238 confirmed the K_0 phenotype. In addition, we found the c.1042C>T mutation (*KEL**02N.04 allele) in both the Manaus K_0 and the Vila Velha K_0 patients. **Conclusion:** This report represents the first study of K_0 molecular basis performed in Amerindian-Caucasian descendants from South America.

Introduction

The Kell blood group system was first described in 1946, following a report of a newborn with an anti-KEL1 (anti-K) causing hemolytic disease [1]. It is considered the most important blood group system after the ABO and Rh, and all frequently occurring Kell-specific antibodies must be considered clinically significant [2]. The *KEL* gene, cloned in 1991, is located on the long arm of chromosome 7 (7q33), with its 19 exons spanning 21.5 kb [3, 4]. The molecular bases for most of the Kell antigens have been determined with the exception of KEL5 (Ku) and KEL20 (Km). The *KEL* alleles are inherited in a co-dominant fashion. The common allele, *KEL**02, encodes all high-incidence antigens of the Kell blood group system. At present 35 KEL antigens are known, which may be classified into six antithetical sets of high- and low-prevalence antigens: KEL1 (K) and KEL2 (k); KEL3 (Kpa), KEL4 (Kpb) and KEL21 (Kpc); KEL6 (Jsa) and KEL7 (Jsb); KEL11 (Côté) and KEL17 (Wka); KEL14 (Scan) and KEL24 (Cls); and KEL31 (KYO) and KEL38 (KYOR). In addition, there are 22 independently expressed antigens, four with low prevalence: KEL10 (Ula), KEL23 (Centauro), KEL25 (VLAN), KEL28 (VONG); and 18 high-prevalence: KEL5 (Ku), KEL12 (Boc), KEL13 (SGRO), KEL16 (k-like), KEL18, KEL19, KEL20 (Km), KEL22 (Ikar), KEL26 (TOU), KEL27 (RAZ), KEL29 (KALT), KEL30 (KTIM), KEL32 (KUCI), KEL33(KANT), KEL34 (KASH), KEL35 (KELP), KEL36 (KETI), KEL37 (KHUL) [2, 5–7]. The low-prevalence antigens reflect single-nucleotide polymorphisms (SNPs) in the *KEL* exons, and some of them show ethnic or racial specificity. Prominent examples are KEL1, which is present in 9% of Caucasians and 2% of people of African descent, and KEL6, which is expressed in 19.5% of people with African descent and less than 0.01% of Caucasians. KEL3 antigen is found in 2.3% in Cauca-

sians and is rare among people of African descent; KEL10 is observed in Finns (2.6%) and Japanese (0.46%), and KEL31 has only been reported in Japanese (1.5%) [2, 8–11].

The Kell glycoprotein (CD238) is a member of the neprilysin family of zinc endopeptidases, whose principal function is the activation of bioactive peptides by proteolytic cleavage of larger inactive polypeptides; however, the function of the Kell glycoprotein on RBCs is unknown [8, 9]. While this family of enzymes has distinct substrate specificity, there is an overlap in function, especially between Kell and endothelin-converting enzymes-1 and -2 (ECE-1 and ECE-2). Big endothelin-3 is the preferred substrate for Kell, being nearly 100 times more effective as a substrate than either big endothelin-1 or big endothelin-2. Nevertheless, Kell is also active in big endothelin-1 and big endothelin-2 [8, 12]. Conversely, ECE-1 and ECE-2 prefer big endothelin-1 as substrate but they can also cleave big endothelin-2 and big endothelin-3. As a group, the endothelins play many different physiological roles. Primarily they act in the blood pressure regulation by affecting contraction and proliferation of vascular smooth muscle and by their vasodilator effects via endothelin-mediated release of nitric oxide. The endothelins are also involved in mitogenesis and developmental process by affecting the differentiation and migration of neural crest-derived cells. The role that Kell, as an endothelin-3-converting enzyme, plays in these processes and whether or not it has a complementary role with XK protein is still unknown [8, 9, 12].

Kell protein is mainly present on red blood cells (RBCs) and testes, and in lesser amounts on brain tissue, lymphoid organs (spleen and tonsil), heart, skeletal muscles, and myeloid progenitor cells [10, 13, 14]. Two distinct proteins, Kell and XK, linked by a single disulfide bond, are responsible for the Kell blood group antigen expression. The XK protein spans the RBC membrane 10 times and expresses only one antigen, Kx. The KEL antigens are carried by Kell which is a 93-kDa type II membrane glycoprotein (CD238) with at least five N-glycosylation sites [2, 8–10]. In rare cases, RBCs lack either XK or Kell protein. RBCs that lack XK have the McLeod phenotype, and RBCs that lack Kell protein have the KELnull (K_0) phenotype [2, 10]. The rare K_0 phenotype is defined by total absence of the Kell protein and all KEL antigens on RBCs and occurs by homozygosity or compound heterozygosity for silent alleles at the *KEL* locus. K_0 RBCs have normal discoid shape but a decreased amount of the XK protein is expressed even though Kx antigen expression appears elevated [2, 10]. Although K_0 persons have lost the enzyme active sites, a lack of Kell enzyme activity does not result in a recognizable illness. Compensatory mechanism may be activated in the K_0 phenotype, probably involving the overlapping functions of other neprilysin family enzymes [8, 9, 12]. K_0 persons, however, can produce anti-KEL5 (anti-Ku) antibody after transfusion and/or pregnancy which is associated to severe hemolytic transfusion reaction and perinatal anemia, requiring K_0 blood for transfusion [2, 10, 15, 16]. In contrast, the absence of the XK protein leads to the McLeod phenotype, which is associated with RBC acanthocytosis and late-onset neurologic defects, and with the depressed expression of Kell protein. McLeod persons can produce antibodies to Kx and Km antigens when alloimmunized [17–19].

Although the K_0 phenotype has been known since 1957, the first reports of the K_0 molecular basis occurred only in 2001 [20–22]. At present, 37 K_0 alleles are recognized to abolish the KEL antigens expression, most of them due to stop codon and alternative splicing caused by single nucleotide mutation. These mutations have been detected in different populations reported by Asian, European and North American studies nevertheless none performed in Amerindian-Caucasian descendants from South America [5, 6, 21–30]. In this paper, we reported the IVS16 + 1g>a mutation (*KEL*02N.31* allele) and the c.1042C>T mutation (*KEL*02N.04* allele) in three Brazilian K_0 patients being descendants of Amerindian-Caucasians.

Material and Methods

Samples

The Recife K_0 patient was a 59-year-old woman of Amerindian-Caucasian descent from Northeast Brazil who showed an antibody that reacted against high-prevalence RBC antigens during a pre-transfusion evaluation for orthopedic surgery and was presumed to be anti-KEL5 (anti-Ku). She had prior history of blood transfusion in 2006. Blood samples in EDTA from the patient, her sister, and two nieces were also tested.

The Vila Velha K_0 case was an 86-year-old woman of Amerindian-Caucasian descent from Southeast Brazil who showed an antibody that reacted against high-prevalence RBC antigens during a pre-transfusion evaluation for orthopedic surgery and was presumed to be anti-KEL5 (anti-Ku). She had prior history of nine pregnancies, but none blood transfusion. Blood samples in EDTA from the patient, her sister, a son, and four grand-daughters were also tested.

The Manaus K_0 patient was a 44-year-old woman of Amerindian-Caucasian descent from Amazon region, North Brazil, suffering from severe anemia (hemoglobin = 3.0 g/dl) while receiving anti-tumor treatment. She presented an RBC alloantibody to a high-incidence antigen, which was presumed to be anti-Ku. She received 3 units of K_0 RBCs obtained from the Rare Blood Group Laboratory of Japan Red Cross in Osaka, and no transfusion reaction was observed.

Two other samples with previously known phenotypes K⁺, k⁺, Kp^a–, and Kp^b+ (control 1) and K⁺, k⁺, Kp^a–, and Kp^b+ (control 2) were used as positive controls. The Ethics Committee Board approved the study.

KEL Phenotyping by Serology

K_0 status was identified by standard serologic techniques that included adsorption-elution at the blood centers that referred these samples. Confirmation and further serology were performed at the blood center of the Federal University of São Paulo using standard immunohematological methods, including the RBC alloantibody screening tests performed with two different RBCs panels (DIAMED Latino América, MG, Brazil, and ASEM-NPBI Produtos Hospitalares, SP, Brazil) before, and after treatment with papain, ZZAP (papain and DDT – dithiothreitol), and AET (2-aminoethylisothiuronium bromide) reagents.

KEL Genotyping and Sequencing

Genomic DNA was prepared from peripheral blood using commercial extraction kit (QIAamp DNA Blood mini kit; Qiagen, Hilden, Germany).

KEL genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as previously described [31–33].

To identify the molecular defect, we used sequencing technique covering all 19 coding and exon-intron regions of the *KEL* gene. 19 exons of the *KEL* gene were amplified into 15 segments using previously described methods [21]. The PCR products were separated by 2% agarose gel electrophoresis and purified using a commercial kit (GFX PCR DNA and Gel Band Purification Kit, GE Healthcare, Hatfield, UK). The purified PCR products were then sequenced using forward and reverse sequencing primers at ABI Prism 3100 Genetic Ana-

Table 1. Phenotypes and genotypes of K₀ individuals and their relatives

Samples	KEL phenotype	KEL genotype PCR-RFLP ^a	Molecular defects
Recife K ₀	K ⁻ , k ⁻ , Kp ^{a-} , Kp ^{b-} , Js ^{b-}	<i>KEL</i> *02/02	IVS16+1g>a
Recife K ₀ sister	K ⁻ , k ⁺ , Kp ^{a-} , Kp ^{b+} , Js ^{b+}	<i>KEL</i> *02/02	IVS16+1g>a [†]
Recife K ₀ nieces	K ⁻ , k ⁺ , Kp ^{a-} , Kp ^{b+} , Js ^{b+}	<i>KEL</i> *02/02	None
Vila Velha K ₀	K ⁻ , k ⁻ , Kp ^{a-} , Kp ^{b-} , Js ^{b-}	<i>KEL</i> *02/02	c.1042C>T
Vila Velha K ₀ sister	K ⁻ , k ⁺ , Kp ^{a-} , Kp ^{b+} , Js ^{b+}	<i>KEL</i> *02/02	c.1042C>T [†]
Vila Velha K ₀ son	K ⁻ , k ⁺ , Kp ^{a-} , Kp ^{b+} , Js ^{b+}	<i>KEL</i> *02/02	c.1042C>T [†]
Vila Velha K ₀ grand-daughters	K ⁻ , k ⁺ , Kp ^{a-} , Kp ^{b+} , Js ^{b+}	<i>KEL</i> *02/02	None
Manaus K ₀	K ⁻ , k ⁻ , Kp ^{a-} , Kp ^{b-} , Js ^{b-}	<i>KEL</i> *02/02	c.1042C>T
Control 1	K ⁻ , k ⁺ , Kp ^{a-} , Kp ^{b+}	<i>KEL</i> *02/02	None
Control 2	K ⁺ , k ⁺ , Kp ^{a-} , Kp ^{b+}	<i>KEL</i> *01/02	None

^aAll individuals were *KEL**02.03 negative and *KEL**02.06 negative.
[†]Heterozygous state (wild type/mutant).

Fig. 1. Chromatograms showing the G>A point mutation on intron 16, position +1 downstream of exon 16 (IVS16 + 1g>a mutation) detected at homozygous state on Recife K₀ patient (A), the heterozygous status observed in the sister of the patient (B), and normal GT consensus at intron 16 splice site on wild type *KEL* gene (C). Leu = leucine; R = A or G.

lyser (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). All sequences were analyzed with BioEdit software (Biological sequence alignment editor; Applied Biosystems, Life Technologies).

The c.1042C>T mutation was confirmed by a PCR-*Tsp45I*-RFLP analysis performed as previously described [20]. A 310-bp DNA fragment that includes exon 9 was amplified, and the PCR products from common Kell-type DNA and from DNA containing the c.1042C>T mutation were treated with *Tsp45I* restriction enzyme (New England Biolabs, Beverly, MA, USA). The C1042T mutation abolishes the *Tsp*-45I restriction site.

KEL Antigen Phenotyping by Flow Cytometry

RBC surface expression of K, k, Kp^a, and Kp^b antigens as well as of total Kell glycoprotein was determined by flow cytometry using human monoclonal soroclone anti-K and anti-k (Fresenius, Bad Homburg, Germany), human polyclonal serum anti-Kp^a and anti-Kp^b (DiaMed, Cologne, Germany), and murine monoclonal anti-total Kell glycoprotein CD238 antibody (Thermo Scientific, Waltham, MA, USA) as primary reagents. Patients' frozen RBCs were washed 3× in 3 ml PBS and 2 × 10⁶ / RBC / 50 µl / tube were incubated for 30 min at room temperature with antibodies (anti-K, anti-k, anti-Kp^a, anti-Kp^b – 50 µl; anti-CD238 1:200, 10 µl). FITC-conjugated polyclonal rabbit anti-human Ig (DAKO) and goat-anti-mouse Ig (DAKO) were used as secondary reagents. Data acquisition and analysis in 20,000 events at RBC region were performed using FACScalibur flow cytometer (BD Sciences, San Jose, CA, USA) and Cell-Quest software program (BD Sciences). Previously known K⁻, k⁺, Kp^{a-}, and Kp^{b+} (control 1) and K⁺, k⁺, Kp^{a-}, Kp^{b+} (control 2) RBCs samples were used as positive controls. To evaluate the interference of the freezing-thawing process on RBC surface antigen expression, fresh and thawed RBCs from controls 1 and 2 were tested by staining with anti-KEL reagents and PE-conjugated anti-human glycophorin. There were no significant differences with regard to KEL and glycophorin expressions between fresh and frozen samples.

Results

Recife K₀ Patient

The patient's RBCs lacked all Kell antigens tested for and thus demonstrating KEL phenotype (K⁻, k⁻, Kp^{a-}, Kp^{b-}, Js^{b-}). She presented an alloantibody that was reactive with all RBCs except her own and Vila Velha K₀ RBCs. The antibody was identified as anti-Ku. Her sister's phenotype was positive for KEL2, KEL4 and KEL7, and her RBCs, as well as those of her nieces, were typed as K⁻, k⁺, Kp^{a-}, Kp^{b+}, Js^{b+} by standard serology techniques. The *KEL**02/02 homozygous genotype was identified by PCR-RFLP technique in the patient and her relatives. They were negative for *KEL**02.03 and *KEL**02.06 alleles (table 1).

The g>a mutation on intron 16, position +1 downstream of exon 16, IVS16 + 1g>a mutation, (*KEL**02N.31 allele) was identified by *KEL* sequencing technique at homozygous state on the Recife K₀ patient and at heterozygous state on her sister (fig. 1). No mutation was detected in the patient's two nieces.

To confirm the K₀ phenotype caused by IVS16 + 1g>a, we performed additional phenotyping tests using flow cytometry. The results of flow cytometry are summarized in table 2. Patient's RBCs were negative for all anti-KEL antibodies. In control 1, the expression was positive for CD238 (MFI = 77), KEL2 (MFI = 189.4) and KEL4 (MFI = 114.4) and negative for KEL1 and KEL3. In control 2, all antigens were positive, but KEL3 (MFI = 7.8) (fig. 2). Results of the RBCs phenotyping of the patient's sister were positive for

Table 2. KEL antigens expression by flow cytometry (in MFI) of Recife K₀ patient and control RBCs samples

	K	k	Kp ^a	Kp ^b	CD238
Recife K ₀ patient	7.6	7.6	8.5	8.0	3.7
Patient's sister	7.6	51.0	8.2	48.0	156.0
Control 1	8.0	189.4	9.3	114.4	77.0
Control 2	76.0	50.0	7.8	49.6	77.0

MFI = Mean fluorescence intensity.

Fig. 2. Flow-cytometric graphics showing different anti-Kell antigens expressions on RBCs in MFI (mean fluorescence intensity) histogram. Expressions of the antigens KEL1 (K1), KEL2 (K2), KEL3 (K3) and KEL4 (K4) are shown on controls 1 (C1) and 2 (C2), Recife K₀ patient (A) and her sister (B) RBCs. CD238 expression is presented on Recife K₀ patient (A CD238), her sister (B CD238) and controls 1 (A CD238) and 2 (B CD238) RBCs. None of KEL antigens was detected on RBCs from Recife K₀ patient (A and A CD238), but K2, K4 and CD238 antigens could be observed on patient's sister (B and B CD238), control 1 (C1 and A CD238) and control 2 (C2 and B CD238) samples. K1 antigen is also expressed on control 2 (C2) RBCs.

CD238 (MFI = 156), KEL2 (MFI = 51) and KEL4 (MFI = 48) and negative for KEL1 and KEL3. The expression of KEL2 from patient's sister RBCs (*KEL*02/02N* genotype) was similar to that observed in control 2 (*KEL*02/01* genotype), with MFI of 51 and 50, respectively, and lower than the control 1 (*KEL*02/02* genotype) (MFI of 51 and 189.4, respectively), reflecting the single gene dose in the patient's sister and control 2, and double gene dose in control 1 (table 2). Unfortunately, a patient sample was not available to perform further RNA analysis.

Vila Velha K₀ Patient

These RBCs typed K⁻, k⁻, Kp^{a-}, Kp^{b-}, Js^{b-}. Anti-Ku was identified in the serum. Her sister's RBCs, as well as those of her son and four grand-daughters, were typed as K⁻, k⁺, Kp^{a-}, Kp^{b+}, Js^{b+}. The *KEL*02/02* homozygous genotype was identified by PCR-RFLP technique in the Vila Velha K₀ and her relatives. They were *KEL*02.03* negative and *KEL*02.06* negative (table 1).

The previously reported [21] *KEL*02N.04* allele, c.1042C>T mutation, was identified by sequencing technique at homozygous state in exon 9 and at heterozygous state in her sister and son. The c.1042C>T mutation confirmed by PCR-*Tsp45I*-RFLP was shown

Table 3. The K₀ alleles reported in this study and in the literature

Number	ISBT allele	Mutation ^a	Intron/Exon	Amino acid change	Reference
1	<i>KEL</i> *01N.01	c.1678C>G	exon 15	p.Pro560Ala	25
2	<i>KEL</i> *01N.02	c.244T>C	exon 4	p.Cys82Arg	30
3	<i>KEL</i> *02N.01	IVS3+1g>c	intron 3	Alternative splicing	22
4	<i>KEL</i> *02N.02	c.382C>T c.1790C	exon 4 (exon 17)	p.Arg128Ter	21
5	<i>KEL</i> *02N.03	c.246T>A	exon 4	p.Cys82Ter	21
6	<i>KEL</i> *02N.04	c.1042C>T	exon 9	p.Gln348Ter	21, 25, this study
7	<i>KEL</i> *02N.05	c.2027G>A	exon 18	p.Ser676Asn	21
8	<i>KEL</i> *02N.06	IVS3+1g>a	intron 3	Alternative splicing	21, 24, 25
9	<i>KEL</i> *02N.07	c.574C>T	exon 6	p.Arg192Ter	6, 21
10	<i>KEL</i> *02N.08	IVS5-2a>g	intron 5	Alternative splicing	23
11	<i>KEL</i> *02N.09	c.1377G>A	exon 12	p.Trp459Ter	23
12	<i>KEL</i> *02N.10	c.1420C>T	exon 13	p.Gln474Ter	24
13	<i>KEL</i> *02N.11	c.903delG	exon 8	p.Met301fs	24
14	<i>KEL</i> *02N.12	IVS8+1g>a	intron 8	Alternative splicing	25
15	<i>KEL</i> *02N.13	IVS8+1g>t	intron 8	Alternative splicing	25, 27
16	<i>KEL</i> *02N.14	c.948G>A	exon 9	p.Trp316Ter	25
17	<i>KEL</i> *02N.15	c.1216C>T	exon 11	p.Arg406Ter	25
18	<i>KEL</i> *02N.16	c.1477C>T	exon 13	p.Gln493Ter	25
19	<i>KEL</i> *02N.17	c.1546C>T	exon 14	p.Arg516Ter	25
20	<i>KEL</i> *02N.19	c.2023C>T	exon 18	p.Arg675Ter	25
21	<i>KEL</i> *02N.20	c.1596G>A	exon 15	p.Trp532Ter	5
22	<i>KEL</i> *02N.21	c.1947C>G	exon 18	p.Tyr649Ter	5
23	<i>KEL</i> *02N.22	IVS7-1g>c	intron 7	Alternative splicing	27
24	<i>KEL</i> *02N.23	c.185insT	exon 3	p.Ser62fs	26
25	<i>KEL</i> *02N.24	c.715G>T	exon 7	p.Glu239Ter	26
26	<i>KEL</i> *02N.25	c.1975delG	exon 19	p.Glu659fs	5
27	<i>KEL</i> *02N.26	c.382C>T	exon 4	p.Arg128Ter	30
28	<i>KEL</i> *02N.27	c.730delG	exon 7	p.Leu236fs	28
29	<i>KEL</i> *02N.28	c.230G>T	exon 4	p.Cys77Phe	6
30	<i>KEL</i> *02N.29	c.1664G>A	exon 15	p.Gly555Glu	6
31	<i>KEL</i> *02N.30	c.71G>A	exon 2	p.Trp24Ter	37
32	<i>KEL</i> *02N.31	IVS16+1g>a	intron 16	Alternative splicing	37, this study
33	–	c.184delT	exon 3	p.Ser62fs	29
34	–	c.821G>A	exon 8	p.Leu274Ter	29
35	–	c.1084C>A	exon 10	p.Gln362Lys	29
36	–	IVS11+5g>a	intron 11	Alternative splicing	29
37	–	c.1708G>A	exon 16	p.Val570Met	29
38	–	c.1975delG	exon 18	p.Glu659fs	30

^aAll mutations were reported on *KEL**02 allele, except for c.244T>C, c.821G>A and c.1678C>G that were detected on *KEL**01 allele.

to be in a homozygous mutant pattern (310-bp band) in the Vila Velha K₀, while we identified the mutation in a heterozygous pattern (90-, 220- and 310-bp bands) in her sister and son. The wild-type pattern (90- and 220-bp bands) was observed in all four grand-daughters.

Manaus K₀ Patient

The RBCs from the Manaus K₀ typed K[–], k[–], Kp^{a–}, Kp^{b–}, Js^{b–}. Anti-Ku was identified in the serum. The *KEL**02/02 homozygous genotype (*KEL**02.03 negative and *KEL**02.06 negative) was identified by PCR-RFLP (table 1).

The c.1042C>T mutation (*KEL**02N.04 allele) was identified at homozygous state in exon 9 by *KEL* sequencing technique and confirmed using PCR-*Tsp*45I-RFLP.

Discussion

Kell is one of the most polymorphic blood group systems with 35 described antigens to date. All antigens result from missense single-nucleotide polymorphisms and subsequent single-amino acid substitutions, with the exception of Km and Ku which are likely to be broadly reactive with one or more epitopes on the Kell glycoprotein [2, 5]. The K₀ phenotype, characterized by total lack of the Kell protein and all KEL antigens on RBC surface, is a quite rare phenotype. Therefore, it is a real challenge to accurately measure the frequency of this phenotype even in populations with similar genetic background. Recent data reported that the K₀ frequency is expected to be 0.00228% in Chinese [26] and no more than 0.00017% in Austrian population [25]. These latest findings

showed much lower frequencies than previously estimated in Europeans and Japanese of 0.007% and 0.008%, respectively [34].

Currently, 37 different K_0 alleles in a scarce number of individuals have been recognized to abolish the KEL antigen expression, most of them due to stop codon and alternative splicing caused by single nucleotide mutation. There are also a few examples of nucleotide insertion or deletion, and 7 cases of missense mutation [5, 6, 21–30]. Table 3 lists the known K_0 alleles. Although homozygosity for an inactivating mutation is more common, there are individuals who are heterozygous for different K_0 alleles among K_0 cases reported. Interestingly, there are 7 K_0 alleles that were only detected in K+, k– persons with $KEL^*01/02N$ genotype: c.1678C>G ($KEL^*01N.01$), c.1216C>T ($KEL^*02N.15$), c.2023C>T ($KEL^*02N.19$), IVS7–1g>c ($KEL^*02N.22$), c.1084C>A, IVS11 + 5g>a and c.1708G>A [5, 25, 27, 29]. Around 1–7% of all seemingly K+, k– individuals may be expected to carry an undetected and rudimentary expressed KEL^*02 allele and, as a consequence, discordant phenotype/genotype results [25, 29]. Therefore, the clinical practice based on DNA analysis for blood group antigens requires caution, and the performance of the blood group phenotype prediction should be improved.

At present, there is no published data about K_0 molecular basis in Amerindian-Caucasians from South America. In this study, we have investigated K_0 samples from three non-related individuals and their available relatives to identify which K_0 alleles are found in the Brazilian highly mixed population, which is composed of Native Amerindians, people of African descent, and European Caucasian descendants mainly from Portugal, which was the colonizer of Brazil in the past. We have identified in this study the $KEL^*02N.04$ allele, in which the c.1042C>T mutation generates a premature stop codon. Interestingly, this mutation was first reported by Lee et al. [21] as cause of the K_0 phenotype in a Portuguese Caucasian woman during her second pregnancy. The c.1042C>T mutation in exon 9 converts a CAA codon encoding glutamine, at position 348, to a TAA stop codon. The c.1042C>T mutation was also detected in an Austrian person who presented the K+, k– phenotype, but the $KEL^*01/02$ genotype. The discrepancy between the phenotyping and genotyping results was elucidated by the presence of the c.1042C>T mutation in the KEL^*02N allele [25]. In this study, we have detected the c.1042C>T mutation ($KEL^*02N.04$ allele) in a homozygous state in two non-related Amerindian-Caucasian individuals (the Vila Velha K_0 and the Manaus K_0 patient), and also in a heterozygous state on sister and son of the Vila Velha's K_0 patient. Presently, $KEL^*02N.06$ (IVS3 + 1g>a) is by far the most frequent KEL^*02N allele, observed in at least 19 individuals from different areas around the world [25]. As a consequence of these new findings, $KEL^*02N.04$ (c.1042C>T) has now become the second most frequent KEL^*02N allele with at least 13 allele observations in

10 different persons – 3 homozygous K_0 and 7 heterozygous individuals – from Europe and South America [21, 25].

The second allele identified in this study was the $KEL^*02N.31$ allele which has a splice site mutation (g>a) in the exon 16-intron 16 junction. The IVS16 + 1g>a mutation was identified at homozygous state on the Recife K_0 patient and at heterozygous state on her sister. This mutation, IVS16 + 1g>a, changes the conserved *gt* sequence and disrupts the 5' splice site, introducing an alternative splicing. Based on evidence from *KEL* and other genes with 5' splice site mutations at this position, it is expected that the exon 16 is skipped in the transcript maturation process [21, 27, 35]. The reading frame shift created by skipping the exon 16 probably introduces a downstream premature stop codon (TGA) on exon 17, or simply result in a shortened sequences that may either be unfavorable at the transcriptional or translational level. As previously described, this gene defect type may also express a truncated protein that is initially inserted into the endoplasmic reticulum membrane, but in no case there was any evidence of the presence of a truncated protein on the RBCs of the K_0 individuals, indicating that the truncated proteins are probably degraded intracellularly and are not transported to the RBC surface membrane [21, 27]. Most likely this splice mutation, IVS16 + 1g>a, is responsible for the absence of the KEL antigens normally encoded by KEL^*02 allele as demonstrated by standard serology and flow-cytometric techniques. These interesting new scientific observations were first reported by our group and presented as an abstract at the 52nd ASH meeting in 2010 [36]. Recently, this IVS16 + 1g>a mutation was also detected in another Brazilian Caucasian female by other Brazilian investigators [37].

In conclusion, in this study we describe the K_0 molecular basis in Amerindian-Caucasian descendants from South America. The c.1042C>T mutation ($KEL^*02N.04$ allele) was identified in homozygous state causing the K_0 phenotype in two Brazilian patients and the IVS16 + 1g>a mutation ($KEL^*02N.31$ allele) was defined in homozygous state in the 3rd K_0 patient. This last reported mutation has only been observed in Brazilians so far.

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Disclosure Statement

The authors declare that they have no conflicts of interest relevant to the present article.

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