Characterization of the –148C>T promoter polymorphism in *PKLR*

We identified the –148C>T mutation in the erythroid-specific promoter of *PKLR* in 3 unrelated patients with low PK activity. *In vitro* transfection studies showed that this promoter substitution did not affect promoter activity. We conclude that the –148C>T promoter polymorphism does not cause PK deficiency and, therefore, should be considered a benign polymorphism.

Pyruvate kinase (PK) deficiency is an inherited enzyme abnormality of glycolysis caused by mutations in the gene encoding liver and red blood cell PK (*PKLR*), and an important cause of hereditary non-spherocytic hemolytic anemia. The clinical phenotype is variable, ranging from severe hemolytic anemia to mild asymptomatic cases[1]. Until now, more than 190 mutations in the PKLR gene have been reported (*http://PKLRmutationdatabase.com*), of which only two are located in the erythroid-specific promoter.[1,2]

A third sequence variation in the promoter of *PKLR* concerns a C to T substitution at nt –148 (–148C>T). This change occurs at polymorphic frequencies in the North American (allelic frequency 0.007 – 0.013, Ensemble SNP 8177960), Portuguese (0.017)[3] and Dutch populations (0.031, this study). The functional relevance of the –148C>T change is unknown but of particular interest since DNA sequence analysis[2] of *PKLR* in 2 unrelated patients with low PK activity (Table 1, individuals 1 and 2) displayed no mutations other than –148C>T. In addition, a third carrier of the –148C>T substitution (individual 3) demonstrated PK activity levels lower than expected while being only heterozygous for the c.1529G>A mutation. We, therefore, hypothesized an association between the –148C>T sequence variation and PK deficiency, as recently also proposed by others.[4]

Analysis of the proximal part of the erythroid-specific promoter of *PKLR* (TFSEARCH database)[5] revealed a putative binding site for the transcription factor c-myb (nts –151 to –142). This binding site was disrupted by the –148C>T mutation. Subsequent transient transfection assays[2] using wild-type and mutant promoter reporter vector constructs in K562 cells showed that the –148C>T substitution did not affect *PKLR* promoter activity (Figure 1, panel A). In addition, Electrophoretic Mobility Shift Assay (EMSA) with K562 nuclear extract did not show a specific protein-DNA complex with the wild-type nor with the mutant *PKLR* oligonucleotide probe (*data not shown*). From these results we conclude that the –148C>T substitution in *PKLR* does not affect erythroid-specific gene transcription *in vitro*.

The role of polymorphic DNA sequence variations in determining susceptibility to disease traits is the subject of much research effort, but it often remains unclear whether they are themselves functionally relevant or just linked to another causative mutation.[6,7] We have demonstrated that the –148C>T change is unlikely to affect *PKLR* expression. Furthermore, the possibility of acquired PK deficiency[8] is considered to be highly unlikely in our patients. However, reduced PK activity in our patients could be due to another still unidentified mutation linked to the –148C>T change. Such a mutation could, for instance, reside in an upstream erythroid-specific enhancer element such as the one that has been shown to be essential for high-level PK expression of the rat *Pklr* gene.[9] Therefore, we used RNA from cultured (pro)erythroblasts of this individual to estimate relative transcript levels of both alleles using the c.1529G>A in exon 11 as a marker.[10] These analyses suggest no difference in expression of both alleles (Figure 1, panel B). Hence, *PKLR* promoter activity appears to be unaffected

Table 1. Patient characteristics.

	Sex/ age	HK 1.02–1.58 (U/g Hb)	G6PD 7.1–11.5 (U/g Hb)	PK 6.9–14.5 (U/g Hb)	Allele 1	Allele 2
1	m/35	1.80	10.0	4.0#	–148C>T	normal
2	m/31	2.17	17.0	6.1	–148C>T	normal
3	m/38	1.35	11.8	1.2	–148C>T	c.1529G>A

#external laboratory, reference range: 8.9 – 11.9 U/g Hb.

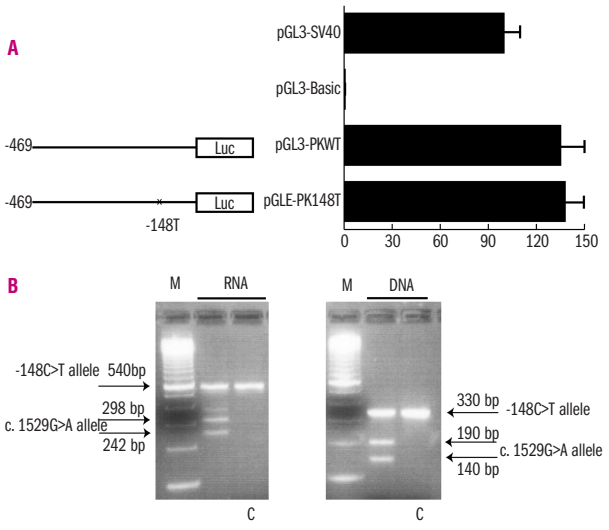

Figure 1. The –148C>T sequence variation in the human *PKLR* promoter does not affect promoter function in K562 cells and is not associated with lower transcript levels in pro-erythroblasts. (A) A *PKLR* pGl3 promoter reporter gene construct (spanning nts –469 to –1) without (pGL3-PKWT) and with the –148C>T mutation (pGL3-PK148T) was transiently transfected in human erythroleukemic K562 cells. Luciferase activities were calculated relative to the pGL3-SV40 positive control vector. pGL3-Basic was included as negative (promoterless) control. Results are the average of four independent experiments with each sample assayed in duplicate. (B) A cDNA fragment encompassing exon 11 was amplified from total RNA obtained by *in vitro* production of (pro)erythroblasts from patient 3 (Table 1) and a control subject (C).[10] A 1:10 diluted sample of this RT-PCR product was amplified in a second round of PCR (three cycles) and subsequently digested with *Sty*I. As the c.1529G>A change creates a unique restriction site for this enzyme, RT-PCR products amplified from transcripts of the patient's c.1529A allele are cut into fragments of 298 and 242 bp whereas RT-PCR products from the patient's c.1529G allele remain uncut (540 bp). The combined intensities of the 242 and 298 fragments are approximately equal to the intensity of the 540 bp fragment, indicating no difference in expression of both alleles. Equal allelic amounts of exon 11, as amplified from the patient's genomic DNA, served as a control. DNA-PCR product of the patient's c.1529A allele are cut into 190 and 140 bp fragments whereas the wild-type allele remains uncut (330 bp). M, molecular mass marker; C, control subject.

by the –148C>T mutation, nor by any other putative mutation linked to this change. The –148C>T mutation in the erythroid-specific promoter of *PKLR* should, therefore, be considered a benign polymorphism. The basis for the lowered PK activity in the 3 individuals presented here thus remains to be established.

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Molecular studies reveal a concordant *KEL* genotyping between patients with hemoglobinopathies and blood donors in São Paulo City, Brazil

Kell is the most important blood group system after ABO and Rh because all frequently occurring Kell-specific antibodies must be considered clinically significant. The highly immunogenic Kell antigens are usually involved in red cell (RBC) alloimmunization that can cause either hemolytic transfusion reactions or perinatal hemolytic disease. Patients with hemoglobinopathies such as sickle cell disease (SCD) require frequent blood transfusions. Therefore, they often become alloimmunized and produce antibodies to low-prevalence Kell antigens, especially KEL1.[1-4] The Kell low-prevalence antigens are expressed due to single-nucleotide polymorphisms in the *KEL* exons and some of them show ethnic or racial specificity. KEL1 is present in 9% of Caucasians and 2% of those of African descent, KEL6 is expressed in 19.5% of Africans and in less than 0.01% of white Caucasians, while KEL3 is found in 2.3% in Caucasians and is rare among Africans.[1] The incidence of alloimmunization, although multifactorial in etiology, is particularly high in SCD patients when compared with other multitransfused patients, primarily due to the disparity of phenotyping pattern distribution which is partly caused by ethnic or racial differences between the blood donor population and the SCD patient population.[2,4] Extended antigen-matched RBC transfusion has been recommended for reducing the incidence of alloimmunization in patients with SCD. This practice adds greatly to the cost of providing blood components to these patients and can cause difficulties in finding RBC units for all patients.[2,3] In order to evaluate differences in KEL genotyping distribution between blood donors and patients with SCD, and the benefit of providing extended antigen-matched RBC transfusion in multitransfused patients, we investigated the frequencies of different *KEL* genotypes in patients with hemoglobinopathies and blood donors in São Paulo City, the largest city in Brazil. The Ethics Committee Board approved the study.

Aliquots of EDTA-anticoagulant peripheral venous blood were collected from 108 Brazilian patients of African descent with hemoglobinopathies and 205 randomized blood donors. Genomic DNA was isolated from whole blood using a commercial kit (QIAamp DNA Blood mini kit, Qiagen, Hilden, Germany). Employing polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique, we performed the *KEL*1,2*, *KEL*3,4,21* and *KEL*6,7* genotyping as previously described.[5-7] The differences between the *KEL* genotype frequencies in the two groups were analyzed by Fischer's exact test. p values of 0.05 were considered statistically significant. The results of the frequencies for *KEL*1,2*, *KEL*3,4,21* and *KEL*6,7* polymorphisms are summarized in Table 1. The *KEL*1/2* genotype was found in 6.5% (7/108) of patients with hemoglobinopathies and in 6.3% (13/205) of blood donors. The *KEL*3/4* genotype was observed in only 1.0% (2/205) of blood donors. The KEL*6/7 genotype was found in 6.5% (7/108) of patients with hemoglobinopathies and in 4.4% (9/205) of blood donors. We also found 1 (0.5%) donor with the *KEL*6/6* genotype. No *KEL*21* allele, *KEL*1/1*, or *KEL*3/3* genotypes were identified among the 313 subjects. There was no statistically significant difference between the frequencies of *KEL*1/2*, *KEL*3/4* and *KEL*6/7* genotypes seen in