Compound heterozygosity in *PKLR* gene for a previously unrecognized intronic polymorphism and a rare missense mutation as a novel cause of severe pyruvate kinase deficiency

Pyruvate kinase deficiency (PKD) is the most common glycolytic enzyme deficiency causing congenital non-spherocytic hemolytic anemia with a variable clinical severity.[1] Approximately 200 mutational variants have been described in the *PKLR* gene with most being in the coding sequence but also in consensus splice sites, and deletions in the 5'UTR involving the GATA1 binding site or of whole exon 11.[1-3] However there are many cases suspected to have PKD on clinical grounds but with only a single mutant allele identified. Here we report a case of PKD caused by novel compound heterozygosity of a missense mutation in exon 7 (NM_000298.5:c.994 G>A) and an intronic mutation (c.283+109C>T, intron 2) causing rapid mRNA degradation (Figure 1).

Case History: The proband, an African American female, now 10 years old, was born at 26-week gestation to a 35-year-old mother with a history of one other living child and 5 miscarriages/abortions. The mother had a normal prenatal ultrasound. The infant was born via cesaeran section due to labor arrest. The birth weight was 900 grams and the hemoglobin (Hb) was 42g/L with a nucleated red cell count (NRBC) of 14.4×10^9/L. The post-natal course was complicated by respiratory distress syndrome, an intraventricular hemorrhage and retinopathy of prematurity. The baby's blood type was A Rh positive and a direct antiglobulin test was negative, while the mother's blood type was AB Rh positive and the antibody screen was also negative. She received eight transfusions during the two months stay in the nursery. Eight weeks after discharge, she was re-admitted (08/27/2008) due to pallor, Hb of 33g/L and decreased activity; the tip of the spleen was palpable and the liver edge was 3 cm below the costal margin. The blood counts are listed in Table 1. A peripheral blood smear showed polychromasia, macrocytic and microcytic red blood cells (RBC), some nucleated RBC (NRBC) and some Howell-Jolly bodies. She was transfused with packed red cells and has remained transfusion dependent since then. Red cell enzyme testing suggested PK deficiency but clinical mutation testing identified a single heterozygous mutation (c.994G>A/pGly332Ser) in the *PKLR* gene (courtesy: JS Friedman; Scripps Clinic, La Jolla, USA). Repeat *PKD* mutation analysis in 2016 confirmed the original c.994G>A mutation, but no other mutations were identified in the *PKLR* gene. No mutations were identified in the genes causing congenital dyserythropoeitic anemias (CDAs: *C15orf41, CDAN1, SEC23B, KLF1, KIF23, and GATA1*).[4]

Thus with parental consent, whole exome sequencing (WES) studies were done on the patient and the mother (Otogenetics, Atlanta, GA).

The biological father could not be tested adequately. The bam/vcf files were analyzed using the integrated genomic viewer and wAnnovar from Broad Institute (*http://wannovar.wglab.org*).

WES confirmed the c.994G>A/pGly332Ser mutation (*rs773626254*) in the *PKLR* gene in the child but not in the mother; thus we assumed this mutation to be either inherited from her father or acquired *de novo*. WES did not identify any SNVs or in/dels in noncoding regions (5'- and 3'-UTRs), extending up to 300bp upstream of 5'-UTR in the patient and the mother. The patient but not the mother also had concurrent *G6PD* mutations, c.376A>G and c.202G>A. WES did not identify any known pathogenic mutations associated with other congenital hemolytic anemias and CDAs. The *PKLR* c.994G>A mutation (minor allele frequency [maf] 0.00006/ExAC Broad Institute) affects one of the highly conserved glycine residues on the β6 strand inside the hydrophobic core of the A domain and results in a highly reduced catalytic activity of the enzyme with low heat-stability.[5] Homozygotes and compound heterozygotes of this mutation have severe transfusion-dependent anemia.[6,7] WES did not identify any of the previously known *PKD* mutations in the mother. However, the child and mother shared 3 mutations in the intron 2 of the *PKLR* gene, c.283+59T>A (*rs8177963*), c.283+109C>T (*rs8177964*) and c.284-114A>G (*rs8177970*); all were validated with Sanger sequencing (Figure 1A).

We hypothesized that these mutations may be located within the junctional complex of the spliceosome, thereby interfering with splicing. *In silico* analysis using SpliceAid2 (*www.introni.it/spliceaid.html*) suggested that c.283+109C>T mutation (maf 0.039/1000G) can result in rapid mRNA degradation through binding of hnRNPs to the mutant motif AUUUA (wild-type: AUUCA; Figure 1B).[8] Additionally, motifs AUUUAUUUA and UAUUUA generated as a result of the same mutation have been reported previously as sites for mRNA degradation.[8,9] Secondary analysis using MutationTaster (*http://mutationtaster.org/*) also suggested the same mechanism.

The mother had low normal hemoglobin (Table 1); the red cell morphology on the smears was normal as well as the red cell deformability pattern evaluated using

Table 1. Blood counts and clinical enzyme test results.

Date of Sample	WBC $\times 10^9$/L	RBC $\times 10^{12}$/L	Hb g/L	Hct %	MCV fL	RDW	NRBC $\times 10^9$/L	Retics %	ARC $\times 10^6$ counts/L	Pyruvate Kinase U/g Hb (Ref range 6.7 - 14.3)	Hexokinase U/g Hb (Ref range 0.8 -1.9)
At birth (4/4/2008)	*19.0	*1.01	*42	*13.3	131.7	18.9	14.4	–	–		
Day 3 post-natal 4/6/2008	*63.7	4.34	*140	*41.8	96.3	26.8	33.6	15.5	672,700		
16 weeks of age 8/27/2008	15.7	1.12	33	10.9	97.3	24.3	–	21	–		
14 weeks Post-splenectomy 11/5/2018	15.7	2.41	*79	25.9	107.5	21.7	1.02	*74.5	*1,805,500	6.0	7.
Mother	11	4.21	122	36.4	86.5	14.0	0.0			5.3	1.4

WBC: white blood cell; RBC: red blood cell; Hb: hemoglobin; Hct: hematocrit; MCV: mean cell volume; RDW: red cell distribution width; NRBC: nucleated RBC; Retics: reticulocytes, ARC: absolute reticulocyte count. (pyruvate kinase and hexokinase activity measurements were done to Mayo Medical Laboratories, Rochester, USA).

Ektacytometer (Technicon), the red cell band 3 content evaluated by eosin maleimide fluorescence and the surface markers CD44, CD47 and CD71 tested using methods previously described.[4,10] The PK activity in the mother was reduced by 50% (Mayo Medical Laboratories, Rochester, USA) suggesting that the c.283+109C>T mutation is indeed pathogenic.

Laboratory confirmation of splicing disorders is complex when the mutations involve potential junctional complex sites or when they are deep intronic. Experimental approaches include – 1) testing for nonsense mediated decay, 2) allele: specific expression; 3) allele-specific complementary DNA (cDNA) sequencing or 4) the more complex mini-gene construct approach.[11,12] *In silico* analyses can guide the choice. In this case we elected the simplest of the approaches namely the allele-specific sequencing based on the absence of any of the known *PKD* linked mutations in the mother and in effect there was a naturally occurring "mini-gene" construct present in her.

At the molecular level, a combination of forward and reverse primers on exon 1, 2, 3 or 4 and intron 2 (Figure 1C; Supplementary Data) resulted in normal length Polymerase chain reaction (PCR) products using the cDNA template. PCR using reverse primer located on intron 2 did not generate any product, suggesting either absence of intron 2 in messenger RNA (mRNA) or unstable mRNA. Since the patient shared the intron mutations with the mother but not the exon 7 mutation, we expect the intron 2 mutations and exon 7 mutation to be present on opposite alleles. We amplified and sequenced the region around the exon 7 mutation on genomic DNA (gDNA) and cDNA for both patient and mother. Sequencing of gDNA showed a single peak for the normal C-allele at c994 in the mother and in the child two equal strength signals for the normal C- and mutant T-

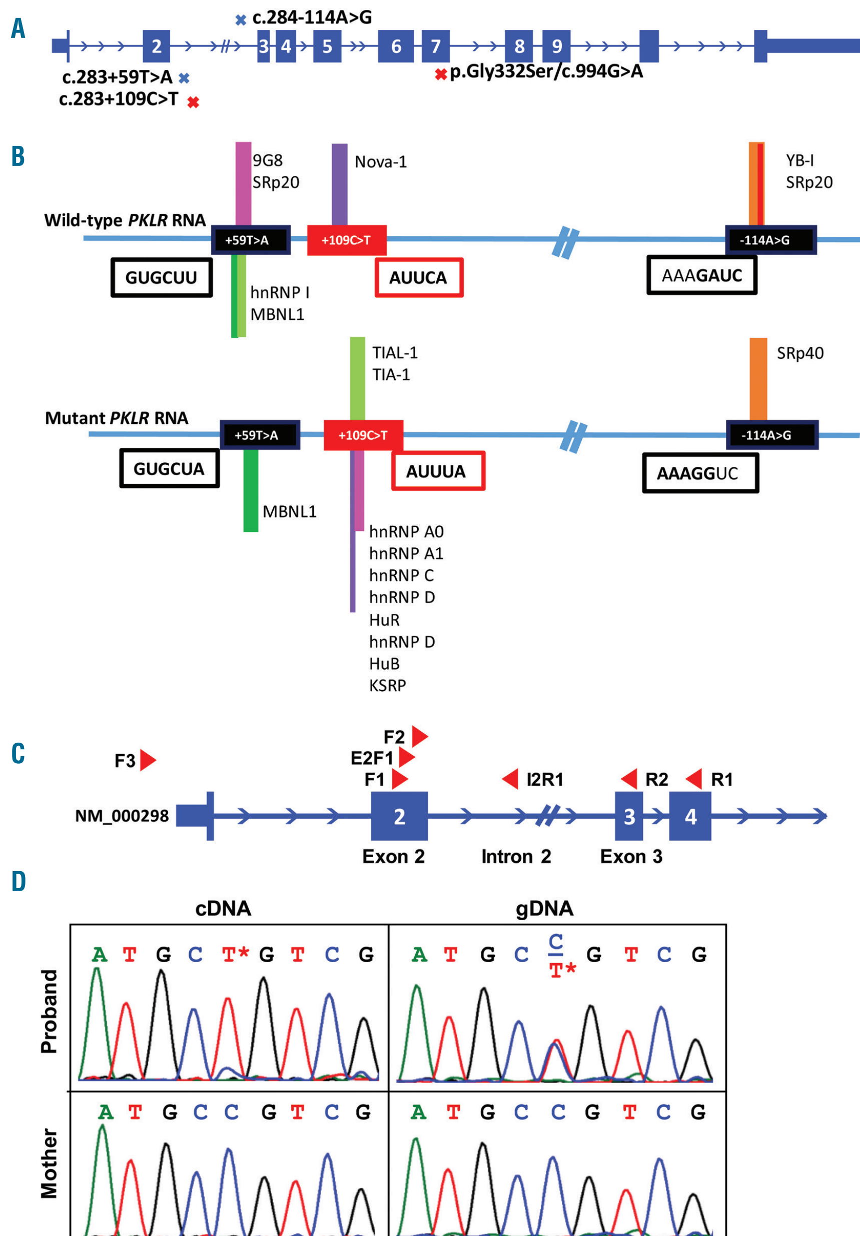

Figure 1. Overview of the PKLR mutations identified in the patient and the experimental design and findings. A. Graphical representation of the mutations present in the proband in the *PKLR* gene. B. *In silico* analysis of all three intronic mutations using SpliceAid2 predicts unique protein binding motifs in WT (left) as compared to mutant (right) RNA. C. Schematic presentation of intron 2 mutations and the PCR primers used to test the *PKLR* transcript. D. Sanger sequence of the cDNA reverse transcribed from mRNA (left) and genomic DNA (gDNA) (right) around the exon 7 mutation of the *PKLR* gene. The proband shows a predominantly mutant allele for the exon 7 mutation (top left) in the cDNA whereas an equal signal for mutant and wild type alleles was identified on gDNA (top right), as compared to the mother with only the wild-type allele (bottom) on both cDNA and gDNA (bottom).

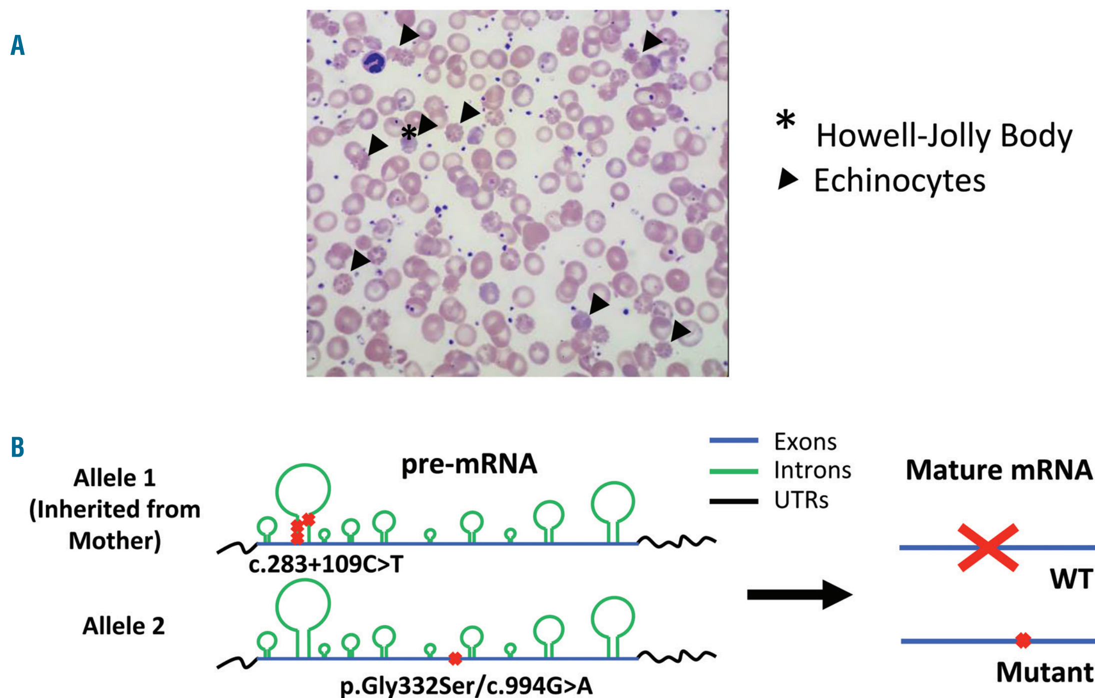

Figure 2. Red cell morphology and model of the *PKLR* transcription in the patient. A. Post-splenectomy peripheral blood smear on the proband; *red cell with Howell-Jolly body, arrows point to echinocytes; B. Model of the *PKLR* transcription in our patient (WT: wild type).

alleles. Allele-specific sequencing of cDNA around the same region in the proband showed predominantly the mutant T-allele with the signal for wild type C-allele at a background level (Figure 1D). The near absence of wildtype cDNA for the 'normal' exon 7 in the patient, indicates rapid mRNA degradation of the "normal allele".

The child underwent splenectomy on July 30, 2018, and for the first time since birth she is transfusion free. Blood counts and clinical enzyme testing at 14 weeks post- splenectomy are listed in Table 1; PK activity was 6.0 U/g Hb (range 6.7-14.5) and hexokinase (HK) activity was 7.5 U/g Hb (range 0.8-1.9) (Mayo Laboratories, Rochester, USA).

The precise diagnosis of severe congenital hemolytic anemias including glycolytic enzyme deficiencies is often complex because of the need for intrauterine transfusions for fetal hydrops or emergent transfusions immediately after birth.[1,4,13,14] In such cases the contamination of transfused red cells often prevents a correct diagnosis using solely clinical red cell enzyme testing on patient cells. Another contributor to the conundrum is the extremely high reticulocyte count and the continued presence of circulating NRBCs. It is not uncommon to see minimally reduced PK activity post splenectomy because of the extremely high reticulocyte counts.[15] The recent PKD natural history study proposed a strategy to normalize the enzyme activity values - "The normalized PK activity was calculated as $[(P_{Kobs} - PK_{LL}) \times 100]/(PK_{UL} - PK_{LL})$ where P_{Kobs} is the observed PK enzyme value, and PK_{LL} and PK_{UL} are the lower limit and upper limit of the reference range, respectively".[1] The study noted normalized PK enzyme activity ranging from -486% to +118% in PKD cases (n=107), confirmed by mutation analysis. Consistent with this, the normalized value for PK activity in our patient using the above formula is -9.2% while the normalized value for HK is 609%, *i.e.* the PK activity in the patient is severely reduced relative to the high reticulocyte count (reticulocyte counts >70%). In contrast the normalized PK and HK activities in the heterozygous mother were -18.42% and +55% respectively. Thus concurrent measurements of PK and HK activities can separate PK deficient anemic patients from heterozygous parents.[13]

Molecular testing offers a solution for reaching a correct diagnosis but is restricted by protocols that include only previously known mutations, may not include junctional/deep intronic regions and large insertions/deletions require separate platforms. Splicing mutations are being increasingly recognized as disease causing.[11] Here we describe a novel mechanism of rapid mRNA degradation as a cause of PKD, confirmed by allele-specific cDNA experiments in the patient's red cells. It should be noted that in the patient, mutation analysis on three different occasions identified only one previously known mutation causing PKD. Other causes of congenital hemolytic anemia (CDA, other glycolytic enzyme disorders, hemoglobinopathies and membranopathies) were excluded by multiple levels of investigation.

Taken together the evidence presented shows that the severe PKD in this child is caused by the combined effect of two very rare mutations (Figure 2) - exon 7 mutation (c.994G>A; *de novo* or inherited from the father) and the intron 2 mutation (c.283+109C>T PKLR^{Detroit}) causing rapid mRNA degradation from the mother.

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