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Alu-Element Insertion in *PKLR* Gene as a Novel Cause of Pyruvate Kinase Deficiency in Middle Eastern Patients

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Abstract

Pyruvate kinase deficiency (PKD) is the most frequent red blood cell enzyme abnormality of the glycolytic pathway and the most common cause of hereditary nonspherocytic hemolytic anemia. Over 250 *PKLR*-gene mutations have been described, including missense/nonsense, splicing and regulatory mutations, small insertions, small and gross deletions, causing PKD and hemolytic anemia of variable severity. Alu retrotransposons are the most abundant mobile DNA sequences in the human genome, contributing to almost 11% of its mass. Alu insertions have been associated with a number of human diseases either by disrupting a coding region or a splice signal. Here, we report on two unrelated Middle Eastern patients, both born from consanguineous parents, with transfusion-dependent hemolytic anemia, where sequence analysis revealed a homozygous insertion of AluYb9 within exon 6 of the *PKLR* gene, causing precipitous decrease of *PKLR* RNA levels. This Alu element insertion consists a previously unrecognized mechanism underlying pathogenesis of PKD.

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AUTHOR CONTRIBUTIONS

H.L., L.D., K.Z., R.J.H., and T.A.K. designed and performed research and analyzed data, X.L., J.D., J.G., S.C., K.G.S., M.M.H. performed research and analyzed data, and all authors contributed to the writing of the manuscript.

DISCLOSURES

The authors declare no conflicts of interest.

Keywords

Pyruvate Kinase Deficiency; PKLR; Hemolytic Anemia; AluYb9; Insertion Mutation; Transposable Element; Retrotransposon

Pyruvate kinase deficiency (PKD) is the most frequent red blood cell (RBC) enzyme abnormality of the glycolytic pathway and the most common cause of hereditary nonspherocytic hemolytic anemia (Koralkova, van Solinge, & van Wijk, 2014). Its incidence has been estimated to be 1 in 20,000, especially in regions where consanguinity is common (Beutler & Gelbart, 2000; Carey et al., 2000). PKD is inherited in autosomal recessive manner occurring as result of homozygous or compound heterozygous mutations affecting both alleles in *PKLR* gene (MIM# 609712, NM_000298.5). Over 250 mutations have been described in patients with PKD causing variable severity of hemolysis (Canu, De Bonis, Minucci, & Capoluongo, 2016). Most cases are due to missense mutations, but small and gross deletions, small insertions, nonsense mutations, splice site alterations, and mutations that disrupt the erythroid specific promoter have been reported. We describe here a previously unrecognized mechanism of gene pathology underlying PKD, caused by the insertion of an Alu retrotransposon in the *PKLR* coding sequence.

Approximately 45% of the human genome is comprised of transposable elements (TEs). Alu elements are the most abundant mobile DNA sequences in the human genome, contributing to almost 11% of its mass (Deininger, 2011; Lander et al., 2001). These retrotransposons have contributed significantly to evolution and human diversity by causing gene rearrangements (Ayarpadikannan & Kim, 2014; Chenais, 2015). Alu insertions particularly have been associated with a number of human diseases either by disrupting a coding region or a splice signal (Hancks & Kazazian, 2016).

The human subjects for this study were tested for diagnostic purposes after obtaining informed consent. Genetic counseling of these individuals, both prior to sampling and after genetic testing results, was provided. The proband was a 16-month-old male born to consanguineous parents, in Saudi Arabia. He was diagnosed with neonatal jaundice and severe hemolytic anemia since birth, requiring regular blood transfusion. There were no known family members with anemia requiring transfusion. He had negative direct antiglobulin test, normal hemoglobin electrophoresis as a newborn prior to any transfusions, normal sequencing and deletion/duplication analysis of the alpha and beta globin genes. The patient's RBC phenotypic analysis was challenging since most of the circulating RBCs were donor cells. His peripheral blood smear revealed mild aniso-poikilocytosis and polychromasia reflecting reticulocytosis (Supp. Fig. 1). The hematologic values and the results of the measurement of peripheral blood PK enzyme activity in the proband and his parents are summarized in Supp. Fig. 1. The proband's PK activity level was detected as borderline low-normal, based on the laboratory reference range, reflecting the presence of donor RBCs. The PK activity measured in the peripheral blood of the mother was low, as expected for heterozygous carriers of PK deficiency; the father was not tested.

After informed consent was obtained, genomic DNA was isolated from blood of the proband and the parents. Using this genomic DNA, we performed mutation analysis using a

previously validated Hereditary Hemolytic Anemia (HHA)/Congenital Dyserythropoietic Anemia (CDA) Next Generation Sequencing (NGS) Panel developed at Cincinnati Children's Hospital Medical Center (Supp. Materials). HHA/CDA NGS panel identified a paternally inherited heterozygous splice site variant in *ANK1* gene (c.1404+15C>T; NC_000008.11: g.41716938G>A; GRCh38) along with a homozygous insertion in exon 6 of *PKLR* gene. To further characterize this insertion, we performed PCR using a set of primers flanking the genomic change in exon 6. Fragment size analysis of this PCR product indicated the presence of a novel fragment. The amplification product for the genomic DNA of the proband was larger (~600 bp) than the expected size of 231 bp (Figure 1A&B). This DNA fragment was sequenced in both directions and an insertion of 367 bp between c.939 and c.940 was identified (c.939_940ins367; p.Ile314fs). Sequencing of the mother's DNA with appropriate primer sets (Supp. Table S1) demonstrated that she was heterozygous for this insertion in *PKLR* (Figure 1C), indicating this allele was an inherited rather than a *de novo* mutation. The insertion occurred at a sequence resembling a LINE-1 endonuclease (EN) cleavage site (Feng, Moran, Kazazian, & Boeke, 1996). Duplication of 15 nucleotides at the site of insertion of the target site was identified, as well as a long poly(A) tract at the end of the inserted sequence (Figure 1D). To determine the nature and origin of the inserted sequence (after omitting the poly(A) tail), a BLAST search was performed against the UCSC human DNA sequence database (University of California at Santa Cruz Genome Browser, <http://genome.cse.ucsc.edu/>). The result indicated that the sequence is an Alu element and further search against the Dfam database of repetitive DNA elements (<http://www.dfam.org/search>) indicated a perfect match with the consensus sequence of the AluYb9 element (Hubley et al., 2016; Roy-Engel et al., 2001) (Supp. Fig. 2).

The insertion of the AluYb9 retrotransposon into exon 6 of *PKLR* gene appears to lead to premature termination of the mutant *PKLR* transcript since a stop codon (TGA) is expected to be readable within frame in the inserted sequence (Figure 1D). The significantly truncated transcript produced would likely undergo nonsense-mediated RNA decay (Lykke-Andersen & Jensen, 2015). To confirm this hypothesis, we used quantitative RT-PCR analysis of sequences within *PKLR* exons 6 and 11 on reticulocyte-derived RNA samples from the proband, and normal control samples (Supp. Materials). We demonstrated that the proband had indeed only a trace amount of mRNA from these exons (10–15% of control levels) (Figure 2A). Normal reticulocytes typically do not express the *PKM* isoform of pyruvate kinase. However, persistent expression of *PKM* isoform was identified in some patients with PK deficiency (Grace et al., 2015; Klei et al., 2017; Lenzner, Nurnberg, Jacobasch, Gerth, & Thiele, 1997; Miwa & Fujii, 1996). In this study, we identified an increased relative expression of *PKM* isoform in the reticulocytes of the proband compared to normal controls. This may be a compensatory mechanism that improves survival in patients with null mutations in the *PKLR* gene (Diez et al., 2005).

In this patient, in addition to the Alu element insertion in *PKLR* gene, we also identified a paternally inherited heterozygous intronic variant in *ANK1* gene (c.1404+15C>T, rs201598401). Heterozygous *ANK1* mutations cause hereditary spherocytosis (Da Costa, Galimand, Fenneteau, & Mohandas, 2013). In silico splicing prediction algorithms suggested that this variant may create a new 5' donor splice site. Further functional analysis was performed using ektacytometry to determine the impact of this variant on RBC

deformability. The father, carrying this variant along with only a carrier state of PK deficiency, had a normal ektacytometry curve (Figure 2B), indicating that the *ANK1* variant c.1404+15C>T is not associated with spherocytosis and should have no significant contribution to the patient's overall clinical presentation. The proband's sample had a borderline increase in osmotic fragility (signified by slightly increased O_{min}) and a slightly decreased EI_{max} , indicating weakened cytoskeleton mechanics, possibly caused by the decreased red cell ATP content due to significant PK deficiency (van Zwieten et al., 2015).

We identified the same homozygous insertion in exon 6 of the *PKLR* gene in a second unrelated patient, an 18-month-old Kuwaiti female born to consanguineous parents, who had presented with transfusion-dependent hemolytic anemia since birth. Her sample was submitted for hereditary hemolytic anemia genetic evaluation (by HHA/CDA NGS sequencing) for clinical diagnosis and the insertion c.939_940ins367, p.Ile314fs was identified. Further work-up, as described above, demonstrated AluYb9 as the inserted sequence. The genomic sequence of the *PKLR* exon 6 gene with the AluYb9 insertion has been submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/; accession number MG677548).

Pyruvate kinase deficiency (PKD) is the most common cause of chronic hereditary nonspherocytic anemia with over 250 *PKLR* mutations published in the literature (Canu et al., 2016). Clinical presentation of patients with PKD varies from mild to severe depending on the impact of the mutations on the protein expression, survival, and enzymatic activity. Here, we report on two unrelated children, born in two different countries in Middle East, with severe transfusion-dependent hemolytic anemia, due to pyruvate kinase deficiency caused by homozygous insertion of an Alu element in the exon 6 of *PKLR* gene, leading to premature termination of the transcript. This case further enriches the contribution of transposable elements in human diseases (Hancks & Kazazian, 2016). Alu insertions have been previously reported in the literature as causing human disease; to our knowledge this is the first report of a pathogenic *PKLR* gene mutation from an Alu insertion and its presence in two unrelated families in two different countries in Middle East implicates the possibility of a founder insertion event in the Arab population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Identification and characterization of AluYb9 insertion into exon 6 of the *PKLR* gene

(A) Schematic representation of the insertion of AluYb9 in exon 6 and the location of primers used for nested amplification. (B) Gel picture of the amplified PCR product from the proband's DNA compared to normal control, using forward primer (5'-GACATCGTCTTTGCCTCCTT-3'), within exon 6 prior to the site of insertion, and reverse primer (5'-TTTCTTCTCCTGCCTGCCT-3') within intron 6 of the *PKLR* gene. The product from the proband's specimen was approximately 370 bp longer than the expected 231 bp fragment from the normal control. (C) Gel pictures of amplified PCR products from the mother and proband's DNA compared to a normal control, using primers to amplify the allele with the Alu insertion (primer set 2) and the normal allele (primer set 3). The Alu-specific forward primer 2 (5'-ACTGGTGACCCAAGTGGAGA-3') is located prior to insertion site in exon 6, while the reverse primer 2 (5'-CCGGCCTTTGCTGATGAT-3') is overlapping the insertion site (sketched in red in Figure 1A). This Alu-specific product is predicted to be 241bp (C-left panel). The normal allele forward primer 3 (5'-ACAAGGGCGGAGAGATGAG-3') is located in distal exon 6 and the normal allele reverse primer 3 is located in exon 7 (5'-CAGCCTGGATGACAGAGTGA-3') (sketched in blue in

Figure 1A). The expected product of the normal allele is 778bp (C-right panel). The Alu-specific primers amplify for the mother and the proband only, while the normal allele primers amplify for the mother and the control, demonstrating that the mother is heterozygous for the Alu-insertion. (D) The cDNA sequence of normal exon 6 compared to the mutated sequence containing the Alu element insertion. The arrow in the normal sequence indicates the site of insertion between c.939 and c.940. Target-site duplication of the PKLR gene sequence flanking the integrated DNA is highlighted in blue; the stop codon within Alu-element is highlighted in red; A and B box sequences (RNA polymerase III promoter) are highlighted in green and violet respectively; poly(A) tail is highlighted in yellow.

Figure 2. Functional Analysis of PKLR and ANK1 variants

(A) qRT-PCR of reticulocyte RNA from the proband and normal controls using primers for the *PKLR* sequence before and after the Alu insertion revealed a transcript decreased at the proband's specimen by ~90% compared to the average of controls. The PKM expression of the proband on the other hand was upregulated, likely as a compensatory mechanism. (B) Ektacytometry profile demonstrated slightly increased O_{min} and slightly decreased deformability (EI_{max}) for the proband but not for the father's RBCs compared to control, indicating that the ANK1 variant is not pathologic since the father does not have an ektacytometry curve compatible with spherocytosis. O_{min} corresponds to the osmolality where 50% of the cells hemolyze in the osmotic fragility test and its value is affected by the surface area to volume ratio. EI_{max} is the maximum elongation that the RBCs can achieve under shear stress and relates specifically to the mechanical properties of the cytoskeleton (Mohandas et al., 1982).