Severe congenital hemolytic anemia caused by a novel compound heterozygous *PKLR* gene mutation in a Chinese boy

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To the Editor: Congenital hemolytic anemia (CHA) can be caused by the defect of any component in red blood cell (RBC), including hemoglobinopathies, membrane and cytoskeleton defects, and metabolic enzymopathies etc. And it is characterized by early present normocytic/macrocytic anemia, reticulocytosis, and elevated unconjugated bilirubin.

Here we reported a Chinese Han infant patient with severe transfusion-dependent CHA. Next-generation sequencing (NGS) revealed the co-existence of 2 compound heterozygous mutations of *PKLR* and *SPTA1* genes. *PKLR* gene is located on chromosome 1q22 and encodes pyruvate kinase (PK). PK participates in anaerobic glycolysis providing 50% ATP for mature RBCs. *PKLR* gene misfunction leads to pyruvate kinase deficiency (PKD) with ATP deprivation and shortened RBCs lifespan. And *SPTA1* gene, located on chromosome 1q23.1, encodes α-spectrin, a major component of cytoskeleton. Defects in *SPTA1* gene leads to unstable RBCs membrane, characterized by peripheral accumulated RBCs with abnormal shape, such as hereditary spherocytosis (HS), hereditary elliptocytosis (HE) and hereditary pyropoikilocytosis (HPP). Both of the 2 genes result in diseases inherited in autosomal recessive manner, so clinical symptoms only occur in homozygotes or compound heterozygotes with 2 mutant alleles.

The infant patient we reported was the first child of a non-consanguineous married Chinese woman, with no family history of jaundice or anemia. He was born by cesarean section at 36^{+6} weeks of gestation because of a suspicious intrauterine distress. He was pale and short of breath at birth. Apgar score were 8, 9, 9, and birth weight was 3150 g. Blood test showed his hemoglobin (Hb) was 73 g/L, RBC count was 1.64×10^{12}/L, reticulocyte percentage (Ret%) was 15.3%, and his total bilirubin was 307 μmol/L (ref value: 5–21 μmol/L), indirect bilirubin was 280.8 μmol/L (Ref value: < 17 μmol/L). The patient's blood type was O and Rh D+, and his mother's blood type was A and Rh D+. Phototherapy and whole blood replacement were given. Hb and RBC increased to 119 g/L and 3.88×10^{12}/L after treatment. Then he was discharged from the local hospital.

Two months later, the patient was sent to the emergency unit because of pale skin and reduced movement. Blood test indicated severe normocytic anemia (Hb 41 g/L, MCV 85.8 fl, MCH 29.1pg; WBC and PLT counts were in the normal range) with reticulocytosis (Ret%: 7.47%, Ret count: 0.0956×10^{12}/L). And an emergency red blood transfusion was given. After that, he received blood transfusions almost every month to keep the Hb level remain at 65–75 g/L.

At the age of 3 years, the boy was recommended to our clinic, and still suffered from life-threatening anemia requiring constant blood transfusion every 4 weeks. Further investigations showed that total and indirect bilirubin were increased. Haptoglobin level was 60 g/L. Direct antiglobulin and Ham tests were negative. CD59 and CD55 were normally expressed on erythrocytes. Bone marrow smear showed erythroid hyperplasia. Peripheral blood smear showed that most RBCs were in normal morphology, and RBC fragments can be seen. Activities of G6PD and PK enzymes were normal. But the results were questionable as he had been transfused frequently. Both his parents were asymptomatic with normal blood tests results. No abnormal findings in their peripheral smear. But the activities of their PK enzyme were near the lower limit of normal range.

NGS in a panel of 600 genes for blood diseases was performed in the index patient and his asymptotic parents by Kindstar Global Esoteric Test & Services Co. α/ß-thalassemia and Fanconi anemia were ruled out. And the

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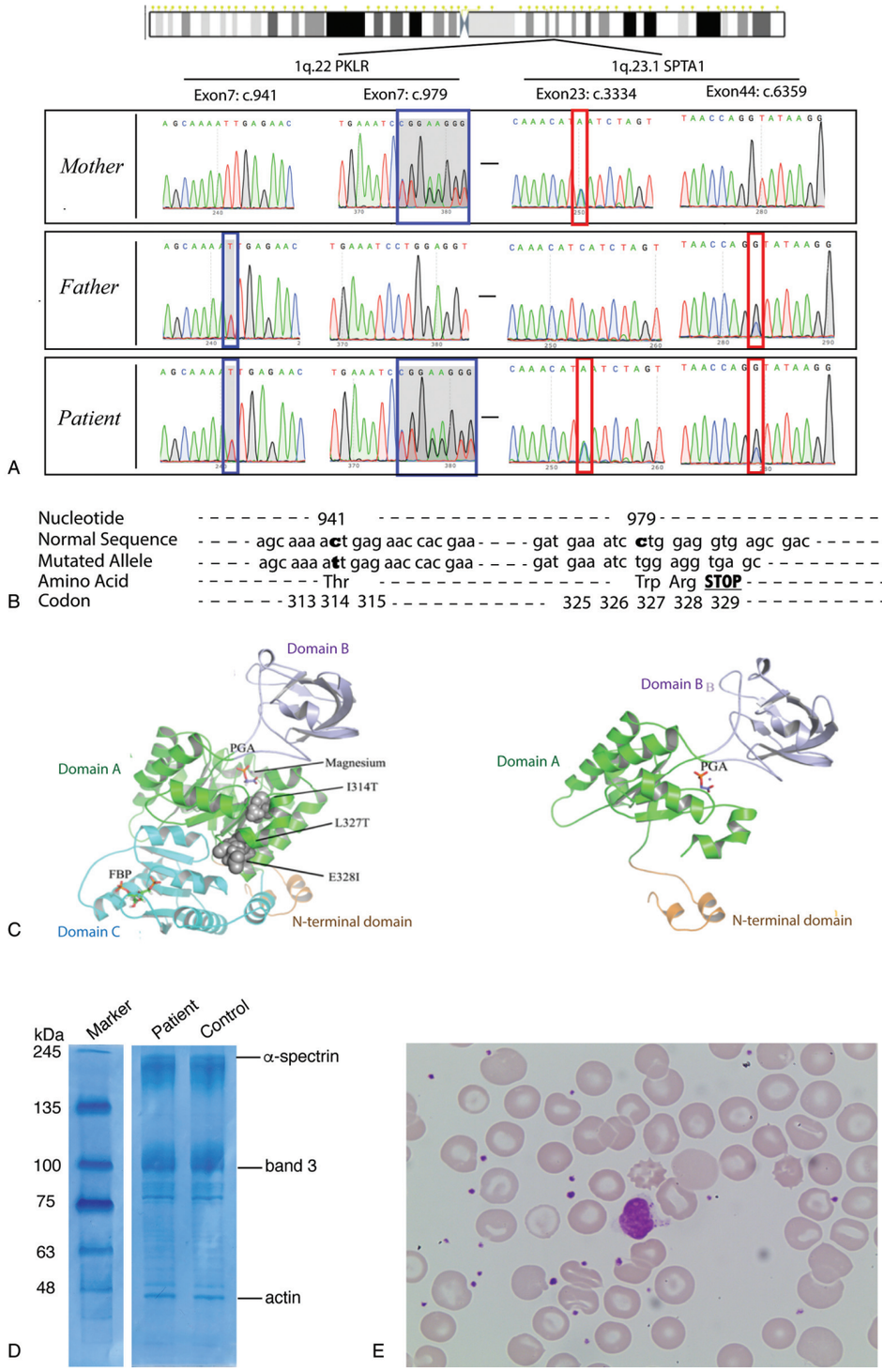

Figure 1: (A) The patient harbors 2 compound heterozygous mutations in *PKLR* and *SPTA1* genes identified by NGS, that is *PKLR*: c.T941C, *PKLR*: c.979delC, and *SPTA1*: c. C6359G, which are inherited from his asymptomatic heterozygous parents. (B) *PKLR* mutations result in amino acid switching and frameshift, and finally lead to (C) nonfunctional and truncated protein by computerized modeling. SDS-PAGE of patient's RBC membrane protein shows (D) no decrease in α-spectrin protein level and peripheral blood smear shows that (E) there is no typical spherocyte and elliptocyte, but stomatocytes could be seen easily.

sequencing analysis identified a combination of 2 compound heterozygous mutations in both *PKLR* and *SPTA1* genes [Figure 1A]. The 2 *PKLR* mutations included one previously described pathogenic non-synonymous single nucleotide mutation, c.941T>C in exon 7, namely Hong-Kong PK mutation,[1] and a novel frameshift deletion, c.979delC, causing false translation after p.327, and we named it as Chengdu PK mutation. And

both of the SPTA1 mutations, that is, c.3334G>T causing p.1112Asp>Tyr and c.6359C>G causing p.2120Thr>Ser, haven't been reported yet. Further family analyze showed that his asymptomatic parents were heterozygotes for both 2 genes. His father was heterozygous for c.T941C:p.I314T in *PKLR* and c.G3334T:p.D1112Y in *SPTA1*, and his mother was heterozygous for the novel c.979delC:p.L327fs mutation in *PKLR* and c.C6359G:p.T2120S in *SPTA1* gene.

The protein variants due to gene mutations were analyzed by computer modeling (Accelrys Discovery Studio 3.1; Accelrys, San Diego, USA). In the aspect of PK, the Hong-Kong PK mutation (c.T941C) caused amino acid Ile switching to Thr at p.314. Ile314 was localized in the hydrophobic core of PK's conservative A domain. The missense mutation led to a hydrophobic Ile to hydrophilic Thr substitution and resulted in the disruption of surrounding hydrophobic interaction network. Besides, the amino acid residue was located between the 313Lys and the 315Glu which were critical for acid-base catalysis and magnesium binding respectively. And as for our Chengdu PK mutation (c.979delC), it was a frameshift mutation and resulted in an early-emerged stop codon at codon 329. After translated, the protein was truncated remaining less than 60% of the normal one. This structure was unstable and lacked many functional sites [Figure 1B-C]. As for α-spectrin protein, it was long and conservative. Both mutations in our case were not in the location with known amino acid molecular structure, so it was hard to predict their effects by the computer modeling method.

At the age of 4 years, because of severe anemia, iron overload and splenomegaly, splenectomy was performed with the preparation of transfusion, as it was an effective therapy for both PKD and RBC membrane defects. The patient's Hb was 103 g/L post-operation. Three months after surgery, his Hb level fell around 70 g/L and a transfusion was given. Before transfusion, his blood sample was taken for blood smear and RBC membrane protein analysis. Now his Hb level kept stably around 80–100 g/L 6 months after splenectomy and 3 months after his latest transfusion. And further follow-up was still going on.

RBC membrane proteins were extracted and resolved through SDS-PAGE [Figure 1D]. Then the gel was stained with Coomassie blue to identify each membrane components. There was no dramatical decrease of α-spectrin content in the patient's RBC membrane compared with normal control when normalized to band 3. The result indicated that although the patient had compound heterozygous mutations in *SPTA1* gene, it did not affect α-spectrin at protein level. And peripheral blood smear was performed at the same time [Figure 1E]. RBCs are larger in shape. Acanthocytes, target cells and stomatocytes could be seen. But there was no spherocytes and elliptocyte, which was typical for *SPTA1* dysfunction, functionally proving that *SPTA1* gene mutations had little effect in the patient's phenotype. So his hemolytic anemia was more likely to be attributable only to PKD.

For the PKD part, 1 missense mutation c.T941C:p.I314T is already proved to be pathogenic in both homozygous and compound heterozygous pattern,[1,2] but does not show any phenotype in heterozygotes. Besides, we first report a novel frameshift mutation, c.979delC:p.L327fs, called Chengdu PK mutation. It causes mistranslation after p327, and even worse, leads to a premature terminal signal appearing just 2 codons after the mutation. The missing parts include the total C domain and more than 1/4 of A domain. Previously, Cotton *et al*[3] reported a homogenous deletion in the *PKLR* gene at c.1010 which caused severe neonatal respiratory distress syndrome and jaundice in a Tunisian boy. The mutation they reported resulted in the termination at codon 340, 11 amino acids longer than ours. Therefore, we believe that Chengdu PK mutation can cause a disastrous damage to PK activity.

As for α-spectrin protein encoded by *SPTA1* gene, it was large and conservative. It containing 20 helical repeat units and inserted functional domains. Disease-causing mutations usually lie in the self-association site (N-terminal) or its vicinity.[4] In our case. None of the *SPTA1* mutations locates in such locations nor known functional domains. And SDS-PAGE proved that the α-spectrin protein didn't decrease in level. Although Poikilocyte were shown on peripheral blood smear after splenectomy, there were no typical spherocyte nor elliptocyte which were related to *SPTA1* mutations. Macrocytes and acanthocytes have been reported to be seen in PKD patients' blood smear after splenectomy. Stomatocytes are characteristic in RBC hydration disorders.[5] In our case, hereditary stomatocytosis has been ruled out by NGS and SDS-PAGE. PKD can cause ATP depriving and Na^+-K^+ pump deactivating, which results in RBC dehydration, contraction, and crenation. So far, there is no article reporting their relationship, further study needs to be done to confirm the mechanism.

In conclusion, the patient we reported suffered from severe hemolytic anemia due to compound heterozygous mutations of the *PKLR* gene. One of the mutation, c.979delC:p.L327fs, is first reported, and we named it as Chengdu PK mutation. The novel mutation resulted in premature stop codon and truncated PK protein. The finding is important to expanse the spectrum of the PKD genotypes.

Declaration of patient consent

We certify that all appropriate patient consent forms were obtained. The patient's legal guardians have given their consent for the case to be reported in the journal, and understand that their names and initials will not appear in the article.

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Conflicts of interest

There are no conflicts of interest.

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