The Egyptian Journal of Medical Human Genetics (2016) 17, 265–270

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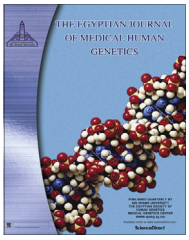

ORIGINAL ARTICLE

Phenotypic and molecular genetic analysis of Pyruvate Kinase deficiency in a Tunisian family

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Received 9 July 2015; accepted 6 September 2015
Available online 26 September 2015

KEYWORDS

Pyruvate Kinase deficiency;
Phenotypic and molecular investigation;
Hemolytic anemia;
Hydrops fetalis;
PKLR mutation

Abstract Pyruvate Kinase (PK) deficiency is the most frequent red cell enzymatic defect responsible for hereditary non-spherocytic hemolytic anemia. The disease has been studied in several ethnic groups. However, it is yet an unknown pathology in Tunisia. We report here, the phenotypic and molecular investigation of PK deficiency in a Tunisian family.

This study was carried out on two Tunisian brothers and members of their family. Hematological, biochemical analysis and erythrocyte PK activity were performed. The molecular characterization was carried out by gene sequencing technique.

The first patient died few hours after birth by hydrops fetalis, the second one presented with neonatal jaundice and severe anemia necessitating urgent blood transfusion. This severe clinical picture is the result of a homozygous mutation of *PKLR* gene at exon 8 (c.1079G > A; p.Cys360Tyr).

Certainly, this research allowed us to correlate the clinical phenotype severity with the identified mutation. Moreover, this will help in understanding the etiology of unknown anemia in our country.

1. Introduction

Red blood cell Pyruvate Kinase (PK) deficiency (OMIM 266200) is the most common enzyme defect in the Embden Meyerhof pathway of glycolysis and one of the most common causes of hereditary non-spherocytic hemolytic anemia in humans. It was described for the first time in 1961 [1]. It is a genetic defect transmitted as an autosomal recessive trait due to several mutations at the Pyruvate Kinase gene (*PKLR*) located on chromosome 1q21 [2]. The coding region is divided into 12 exons, 10 of which are common to the two isoforms, while exons 1 and 2 are specific for erythrocytic (*PK-R*) and hepatic isoenzymes (*PK-L*) respectively [3–5]. The crystal structure of the functional enzyme reveals that *PK-R* is a homotetramer and each subunit consists of four domains

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Peer review under responsibility of Ain Shams University.

http://dx.doi.org/10.1016/j.ejmhg.2015.09.001

(N, A, B and C domain) [6]. Domain A is the most highly conserved whereas the B and C are more variable [7].

At the molecular level, more than 220 different mutations, involving exons, introns and splice sites, have been described so far in association with PK deficiency worldwide with several recurrent mutations (www.lovd.nl.pklr). Among these, three are reported to be the most prevalent in deficient patients in different ethnic groups (1529A, 1456T and 1468T) [8–12].

Clinically, patients manifest a highly variable phenotype. The degree of hemolysis fluctuates from mild or fully compensated forms to whole life threatening anemia necessitating exchange transfusions. In some severe conditions, individuals may die in utero or at birth by hydrops fetalis [13–16].

In Tunisia, PK deficiency is yet poorly documented. Up to date, only one reported case of Tunisian origin was described on 2001 by Cotton et al. [17]. Deficient patients could die by hydrops fetalis or in perinatal period. Moreover some of them spend suffering for a long time without correct investigation. Here in, we report the case of a severe hemolytic anemia due to PK deficiency originated by a homozygous mutation in the *PKLR* gene not yet reported in the Tunisian population.

2. Subjects and methods

2.1. Subjects

This study was carried out on two brothers from consanguineous parents (Fig. 1). The first one IV-2 died few hours after birth by hydrops fetalis. The second one IV-3 who was born three years after, presented with neonatal jaundice and deep anemia necessitating urgent blood transfusion. He was transferred to neonatal intensive care for phototherapy and further biological investigations.

After formal consent, the DNA of the dead baby IV-2 (obtained from DNA bank of the hospital) and blood samples from the second proband IV-3 and his family (I-1, II-2, II-3, III-1, III-2 and IV-1) were requested for further genetic analysis (hemoglobinopathies, enzymopathies and Gilbert disease). This study was approved by the Ethics Committee of Pasteur Institute of Tunis, Tunisia in accordance with The Code of Ethics of the World MEDICAL Association Declaration of Helsinki.

2.2. Methods

2.2.1. Hematological and biochemical analysis

Red blood cell (RBC) indices and reticulocyte count have been obtained automatically by a Coulter Counter (ABX pentra 60 C+, HORIBA Diagnostics). Coombs test, Blood smear, bone marrow examination and other biochemical tests including serum vitamin B12, ferritin level and bilirubin have been carried out according to standard methods. Search for hemoglobinopathies (alpha and beta thalassemia, sickle cell disease) was made using an HPLC analyzer D10 Hemoglobin testing system (BioRad Laboratories, Hercules, CA, USA).

A purified RBC (without leukocytes and platelets) was prepared for PK and glucose-6-phosphate dehydrogenase (G6PD) assays, by passing the whole blood through a column containing alpha-cellulose and sigma-cellulose (Sigma–Aldrich®). Enzymatic assays and estimation of 2,3 bisphosphoglycerate (2,3 BPG) level were performed as described by Beutler et al. [18].

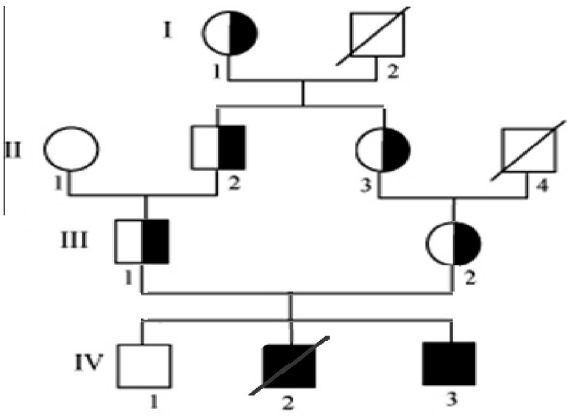

Figure 1 Pedigree of the studied family. The (c.G1079A) mutation was identified at the homozygous state in the proband IV-3 and his dead brother IV-2. It was heterozygous for both parents (III-1 and III-2) and all other investigated members of the family (I-1, II-2, II-3). The brother IV-1 exhibits a normal genotype.

2.2.2. Molecular investigations

Genomic DNA was isolated from peripheral blood leukocytes by phenol/chloroform extraction according to standards protocols. The erythroid-specific promoter and exons of *PKLR* gene, including flanking intronic regions, were amplified by PCR as previously described [19].

The analysis of the A(TA)$_n$TAA motif in the promoter region of the uridin-diphosphoglucuronyl transferase gene (*UGT1-A1*), responsible of bilirubin glucuronidation, was performed as described by Galanello et al. [20]. PCR was performed in 25 µl reaction volume containing 100 ng of genomic DNA, 0.2 mmol/l of each dNTP, 1× PCR buffer, 2.5 mmol/l $MgCl_2$, 0.5 units of Taq DNA polymerase (Roche®) and 10 pmol of each primer (TAF: 5′TCGTCCTTCTTCCTCTCTGG3′ and TAR: 5′TCCTGCTCCTGCCAGAGGTT3′). The PCR cycling conditions included an initial denaturation step of 10 min at 96 °C followed by 35 cycles of 96 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. The run was ended by a final extension at 72 °C for 7 min.

DNA sequencing was performed on an ABI PRISM® 3600 DNA Analyser using ABI PRISM®BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

3. Results

The clinical, biochemical, hematological and molecular investigations of the studied family are summarized in Tables 1 and 2.

3.1. Clinical, biochemical and hematological data

The proband IV-3 presented at birth with severe anemia and neonatal jaundice. He received 12 blood transfusions during the first fourteen months of his life. At the age of three years and before any transfusion, the patient was diagnosed at the

Table 1 Hematological, biochemical data and hemoglobin pattern of the proband IV-3.

		Reference value
Hb (g/dL)	6.8	12–17.5
Retic (10^9/L)	240	25–86
MCV (fL)	82	82–98
MCH (pg)	28.8	27–32
MCHC (g/100 mL)	35.2	32–36
Ferritin (µg/L)	82.5	30–300
Vitamin B12 (pmol/L)	675	150–700
2,3 BPG (µmol/g Hb)	20	7.5–12
Total bilirubin (mg/L)	13	< 10
Hb A (%)	97.5	
Hb A2 (%)	2.5	

Hb, hemoglobin; Retic, reticulocyte; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; 2,3 BPG, 2,3 biphosphoglycerate; Hb A, hemoglobin A; Hb A2, hemoglobin A2.

hematological, biochemical and molecular level. He presented a severe hemolytic anemia with reticulocytosis, normal Mean Corpuscular Volume (MCV), normal ferritin level and a normal Hemoglobin Pattern excluding an iron deficient anemia and thalassemia. The direct Coombs test was negative. Thus, the hemolytic anemia caused by immune system lysis was excluded. Bone Marrow examination smear showed an increased portion of erythroblasts (46%) with no specific dyserythropoiesis.

Biochemical data of the proband indicated that the 2,3 bisphosphoglycerate (2,3 BPG) level is twofold increased comparing to the normal range (20 µg/g Hb; instead of 7.5–12). Further to that, total bilirubin level was high suggesting a possible Gilbert disease (Table 1).

The enzymatic assays showed normal G6PD level but reduced PK activity (Table 2). At this step, our investigation was oriented to the search of Gilbert disease, PK deficiency or their co-inheritance.

The patient IV-2 died few hours after birth by hydrops fetalis. No other clinical and biological data are provided. The PK activities of the parents and the great grand-mother I-1 are reduced. However, the brother IV-1 had a normal PK activity (Table 2).

3.2. Molecular analysis

Molecular analysis was performed for both Gilbert disease and PK deficiency.

Analysis of the promoter region of the *UGT1A1* gene in the proband showed a normal polymorphic sequence $A(TA)_6$-TAA, excluding Gilbert disease. In fact, our molecular data on this syndrome showed the association of a unique polymorphic sequence $A(TA)_7$TAA with Gilbert syndrome among Tunisians (Fig. 2) [21].

The screening of whole *PKLR* gene revealed the presence of a G > A transition at nucleotide 1079 in the 8th exon (p. Cys360Tyr). This mutation was identified at the homozygous state in the proband IV-3 and his dead brother IV-2, whose parents were related. It was heterozygous for both parents (III-1 and III-2) and all other investigated members of the family (I-1, II-2, II-3). The brother IV-1 exhibits a normal genotype (Fig. 3).

4. Discussion

PK deficiency, an autosomal recessive disorder, is probably the most common cause of hereditary non-spherocytic hemolytic anemia, often causing severe anemia from infancy or early childhood [16]. The degree of consanguinity among parents increases the risk of inheriting recessive disorders in their children.

There are many and various mutations in the *PKLR* gene described worldwide (www.lovd.nl.pklr). The present study constitutes a complete phenotypical and molecular investigations of PK deficient patients in Tunisia and represents the first description of the Cys360Tyr mutation at the homozygous state worldwide. However, It was reported once in a compound heterozygous state (Arg479His/Cys360Tyr) by Pissard et al. [13,26].

PK deficiency remains misdiagnosed in our country. Several deficient subjects develop transfusion dependent anemia before to be treated by splenectomy [22,23] without correct diagnosis. The most severe clinical complication of PK deficiency is hydrops fetalis [15,24,25] that was observed in the dead patient IV-2. The proband IV-3 displayed a severe hemolytic anemia requiring regular blood transfusions. Fortunately, he was early diagnosed and had regular medical follow up based on transfusion therapy. The clinical severity of our patients (IV-2 and IV-3) is most likely due to the homozygous

Table 2 Enzyme activity, clinical manifestations and genotype of the studied family.

P	PK activity (IU/gHb) (R.R: 5.2–8.5)	G6PD activity (IU/gHb) (R.R: 5.26–7.94)	Clinical data	Transfusion	Genotype
IV-3	1.75	5.26	Neonatal jaundice and severe hemolytic anemia	Yes	1079A/1079A
IV-2	ND	ND	Hydrops fetalis and neonatal death	No	1079A/1079A
IV-1	6.43	5.5	Normal	No	No mutation
III-1	2.7	6.2	ND	No	1079G/1079A
III-2	2.68	5.8	ND	No	1079G/1079A
II-2	ND	ND	ND	No	1079G/1079A
II-3	ND	ND	ND	No	1079G/1079A
I-1	3.01	5.43	ND	No	1079G/1079A

P, patient; R.R, reference range; ND, no data; PK, Pyruvate Kinase; G6PD, glucose-6-phosphate dehydrogenase.

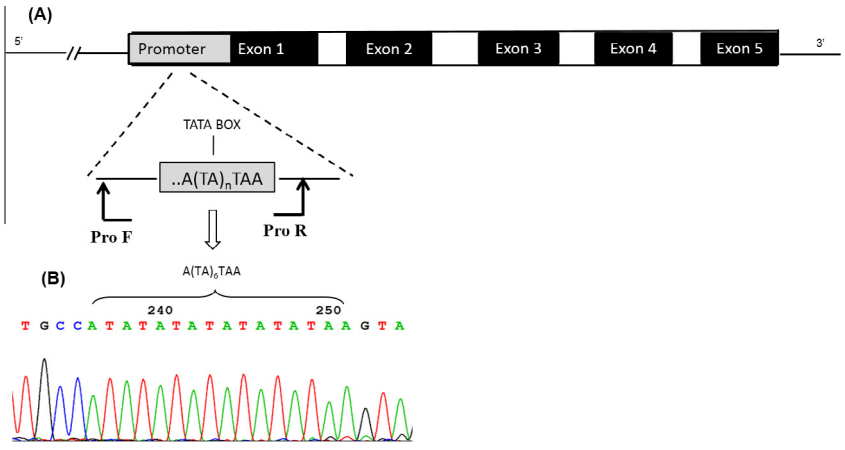

Figure 2 The ultrastructure of *UGT1A1* gene showing the position of primers designed for PCR (A). The DNA sequence analysis of the *UGT1A1* promoter region (B).

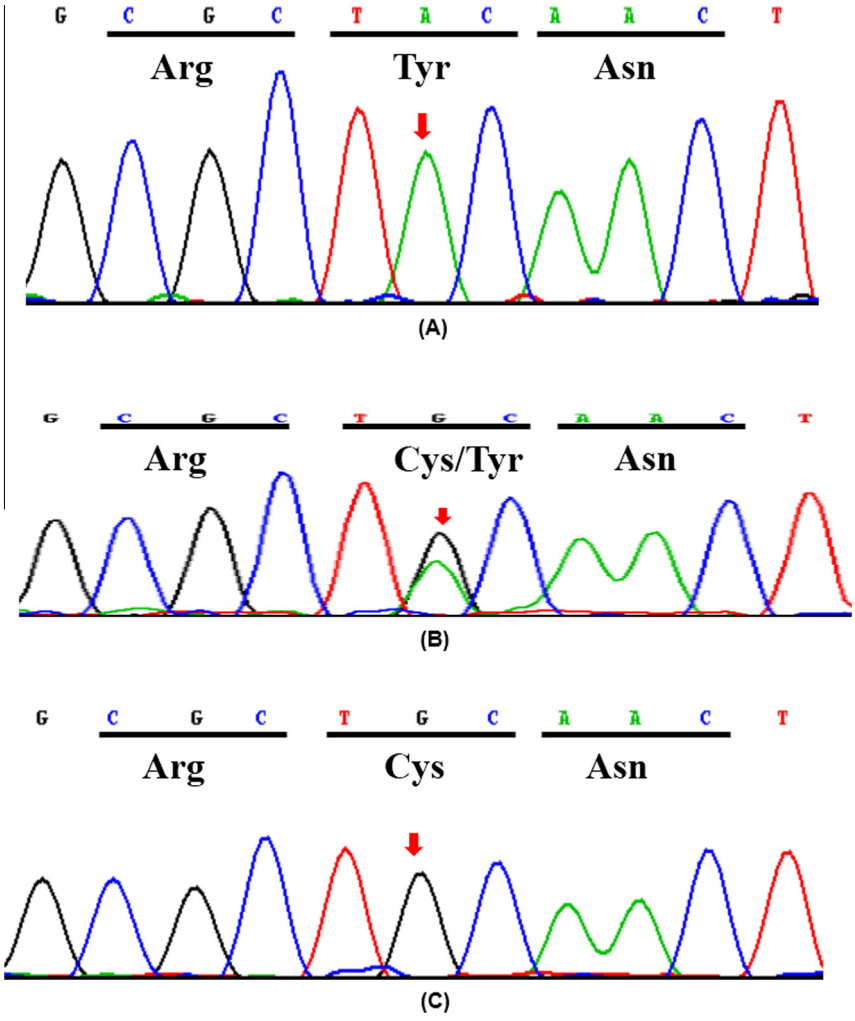

Figure 3 Direct sequencing of exon 8 of *PKLR* gene in the proband and his family. The patients IV-2 and IV-3 appeared to be homozygous for (c.1079A; pCys360Tyr) mutation (A); I-1, II-2, II-3, III-1 and III-2 are heterozygous (B); the brother IV-1 is normal (C).

mutation (Cys360Tyr). It is a disruptive mutation associated with a severe clinical expression. In fact, Pissard et al. in 2006 reported two different patients with different clinical presentation, one of them presented with severe clinical presentation and was compound heterozygous (Cys360Tyr/Arg479His), the second one, although homozygous for

Table 3 Effect of Cys360His and Arg479His mutations in the heterozygous/homozygous states.

Genotype	Number of cases	Hb (g/dl)	Transfusion	PK activity (IU/g Hb)	Clinical manifestations	References
Arg479His/ Cys360Tyr	1	6.4	- Transfusion in utero - Severe anemia requiring 22 packed RBC transfusion during 3 years	–	Acute hemolysis during the third trimester of pregnancy	[24,25]
	2 (twins)	–	–	–	Neonatal death	
Arg479His/ Arg479His	1	10	–	2.7	Neonatal anemia	[24]
Cys360Tyr/ Cys360Tyr	Patient IV-3	6.8	12 blood transfusion	1.75	Severe neonatal anemia and neonatal jaundice	This study
	Patient IV-2	–	–	–	Neonatal death by hydrops fetalis	This study

Hb, hemoglobin; PK, Pyruvate Kinase.

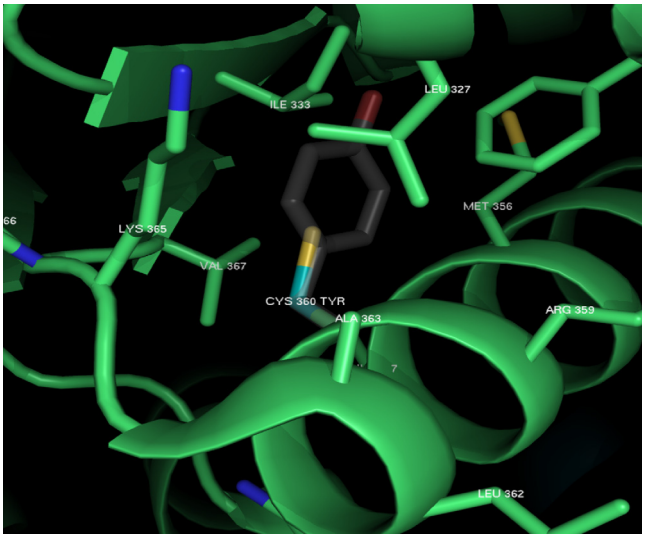

Figure 4 Three dimensional crystal structure of human RPK Cartoon drawing of the vicinity of the p.Cys360Tyr substitution. The side chain of Tyr360 (depicted with dark gray) creates a steric hindrance in the molecule, especially at the contact area of Tyr360 side chain and Leu327 residue. The figure was generated from the atomic coordinates of protein data bank entry 1LIU [6] using the program PyMOL (DeLano WL. The PyMOL molecular graphics system, 2002 on World Wide Web http://www.pymol.org).

another mutation (Arg479His/Arg479His) exhibits a mild clinical presentation (Table 3) [13]. This could lead to the conclusion that Cys360Tyr mutation might be associated with the severe clinical form of PK deficiency even at the homozygous or compound heterozygous states.

The substituted Cys residue at position 360 is located in the A domain (α6) of the PK subunit [6], which is the most highly conserved domain [7]. Moreover, it contains residues involved in the active site. Several studies have demonstrated that mutations in A domain, especially in the homozygous form, are associated with severe hemolytic anemia and necessity of transfusion [6,13,19]. Furthermore, a number of mutations located in the A domain (p.Asp331Gln; p.Gly341Asp and p. Thr384Met) are associated with hydrops fetalis, such as the case of our patient IV-2 [16,24]. These data confirm that the structural alteration of this domain would result in severe PK deficiency. In addition, the molecular modeling of this mutation revealed that the Tyr360 side chain is buried in the hydrophobic core of the molecule. A severe sterical hindrance and unfavorable interactions were created between Tyr360 side chain and Ile333, Met356, Val367 and especially Leu327 side chains (Fig. 4).

The mutation described here is considered as one of the more severe variants. Hence, identification of this type of mutation is essential for accurate antenatal diagnosis. Its presence for at least three generations in a Tunisian family indicates that we are probably in front of an ancient variant whose origin could be explained by a founder effect within the Tunisian population or by genetic flow through population migration as it was assumed for sickle cell mutations and some beta thalassemia alleles [27].

5. Conclusion

The description of these cases of PK deficiency in Tunisia opens the access to the study of all ambiguous anemias at the molecular level mainly at early age. Since only transfusion therapy is available for the treatment of PK deficiency and severe cases may be improved by splenectomy. In the case of the young patient described in this study, as anemia tends to improve and stabilize with maturation, a few years of observation are necessary before making any decision.

Declaration of interest

This study was supported by a grant from The Scientific Research Ministry of Tunisia:

Laboratory of Molecular and Cellular Hematology, Pasteur Institute of Tunis (LR11IPT07).

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Acknowledgements

The authors are grateful to all the members of the staff at the laboratory of molecular and cellular hematology at Pasteur Institute, Tunis, Tunisia for their close cooperation and important contributions.

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