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Co-existence of hereditary spherocytosis and a new red cell pyruvate kinase variant: PK Mallorca

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ABSTRACT

Background and Objectives. **A partial red blood cell (RBC) pyruvate-kinase (PK-R) deficiency was found in a patient with concomitant hereditary spherocytosis (HS) and chronic hemolytic anemia. Clinical, biological and molecular studies were performed in the patient, his parents and a brother, in order to characterize the specific PK-R gene mutation and the inheritance mechanism of the transmission of both red cell defects in this particular family.**

Design and Methods. **Conventional biological studies were used to identify the PK-LR gene mutation responsible for hereditary transmission of PK-R deficiency and HS. The family study was completed with genotypic and RBC membrane protein analyses in the patient and his family.**

Results. **Molecular study of the PK deficiency was performed in all the family members and demonstrated a heterozygous condition for the 1516 G→A (506Val→Ile) mutation at the PK-LR gene in both the patient and his mother. Since this mutation has not been reported previously, it is provisionally named PK "Mallorca". The study of RBC membrane proteins demonstrated the existence of partial band 3 and protein 4.2 deficiencies in the propositus and his father but not in the mother and brother, who were also studied. These results support the dominant mode of inheritance of HS and PK-LR gene in this family.**

Interpretation and Conclusions. **HS and PK deficiency are not exceptional in Spain. The co-existence of both RBC defects in the same patient, however, is very rare; only a few cases have been described to date. Our findings suggest that performing an elementary RBC enzyme survey in all patients with HS would help to determine the real frequency of this apparently rare association.**

Key words: hemolytic anemia, pyruvate kinase deficiency, hereditary spherocytosis, SSCP, PCR-RE

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Hereditary spherocytosis (HS) is a common inherited hemolytic anemia with a relatively high incidence in Spain.[1] It is becoming clear that it is a highly heterogeneous disorder, in terms of clinical expression, inheritance, and underlying genetic defects.[2] Laboratory diagnosis of HS requires the presence of circulating spherocytic red blood cells (RBCs), increased osmotic fragility and, when possible, the demonstration of the underlying membrane protein defect.[3,4] In HS the primary molecular abnormality may affect one or more of the following RBC membrane structural proteins: spectrin, ankyrin, band 3 and protein 4.2. Band 3 or anion exchanger 1 (AE1) is the most abundant protein in the RBC membrane, and between 20% and 40% of HS cases (depending on geographical location) present a substantial, generally around 30%, reduction of this protein.[5] Basically, the inheritance pattern of HS is autosomal dominant and its clinical phenotype is mild to moderate hemolytic anemia in the heterozygote situation. Several mutations leading to band 3 reduction, generally accompanied by a roughly proportional decrease in protein 4.2, have recently been identified in HS.[6] Despite this significant progress in our understanding of the disorder's molecular biology, diagnosis of HS still depends on RBC morphology, standard laboratory tests and careful family studies. Association of HS with other RBC congenital defects is rare but not exceptional; it has been described with β-thalassemia and glucose-6-phosphate dehydrogenase (G6PD),[7-9] and also with congenital RBC pyruvate kinase deficiency.[10]

Congenital pyruvate kinase (PK: EC. 2.7.1.40) deficiency is an enzymopathy of anaerobic glycolysis that essentially differs from HS by the absence of circulating spherocytes, the fact that osmotic fragility tests are normal, and a more severe chronic hemolytic anemia.[11] Clinical manifestations of PK deficiency are, in general, only present in subjects homozygous or *double-heterozygous* for two different gene mutations.[12-19] Heterozygote carriers of the enzyme deficiency are usually devoid of clinical manifestations; some cases, however, exhibit overt neonatal jaundice or chronic hemolytic anemia triggered by special circumstances such as pregnancy, infections or concomitant metabolic diseases.[20]

Since the first description of PK deficiency by Valentine *et al.*[21] and Tanaka *et al.*[22] more than 200 molecu-

lar variants have been identified on the basis of deficient enzyme biochemical properties.[23] The severity of the anemia in the PK deficiency is not always related to the intensity of the underlying molecular defect.[24-29] In Spain, descriptions of PK deficiency are scarce, and practically all of them refer to sporadic clinical cases.[30-31] By using the ICSH recommended procedures for PK deficient enzyme characterization,[32] ten different PK variants associated with chronic non-spherocytic anemia (CNSHA) have been described since 1980.[33,34] More recently, using PCR-RE, PCR-SSCP and gene sequencing procedures,[35] a genotypic analysis was performed in 12 patients with PK-R deficiency and CNSHA, demonstrating that the most frequent mutation is the replacement of cytosine by thymine at 1456 position.[19] This mutation has also been described in Italy,[15] but not in studies performed in northern Europe[36,37] and the USA.[38-39]

To our knowledge, only one case of concomitant PK deficiency and HS has been described to date.[10] In contrast, PK deficiency has been associated with concomitant G6PD deficiency, β-thalassemia, hereditary elliptocytosis and Gaucher's disease.[11] Furthermore, an acquired defect of the enzyme has frequently been reported in a range of hematologic malignancies such as leukemia and myelodysplastic syndromes.[23] In the present study, a partial PK deficiency was found in a patient with HS and moderate hemolytic anemia. Clinical, family, genetic and molecular studies allowed determination of the interrelationship between the two RBC abnormalities in the patient and his relatives (parents and brother) and description of a new mutation at the *PK-LR* gene which has been provisionally designated PK Mallorca due to the geographical origin of the patient. The *PK* gene study performed in both parents demonstrated that the patient and his mother were heterozygote carriers for the new PK mutation.

Clinical history

A 9-year old male, a native of Palma, Mallorca (Balearic Islands) was admitted to our Department for a family study. HS had been diagnosed in his father, who had presented hemolysis with anemia after an acute cholecystitis attack due to gallstones. Family history revealed the existence of chronic anemia in the 77-year old paternal grandmother. The patient had no previous history of anemia or hemolysis, and his mother and brother were clinically and hematologically normal.

Design and Methods

General hematologic data and hemolytic tests

Heparinized and EDTA anticoagulated blood was obtained from the patient and his relatives. RBCs were purified by filtration through cellulose and acellulose columns; RBC enzyme activities [glucose-6-phosphate dehydrogenase (G6PD), pyruvate kinase (PK), hexokinase (HK), phosphohexoseisomerase (PHI), dehydrogenase 6-phosphogluconate (6PGD) and erythrocyte acetylcholinesterase (ACE)] were assayed within 24 hours of blood drawing by standard methods.[3] General hematologic data, reticulocyte count, erythrocyte morphology, osmotic fragility tests (OFT) and acidified glycerol lysis tests (AGLT) were performed by standard methods.[40]

RBC membrane proteins

Contents of each membrane protein fraction were determined by gels of polyacrylamide in denaturalized conditions with SDS-PAGE, following the method of Fairbanks *et al.*[41] in order to quantify the α and β spectrin (bands 1 and 2, respectively) and ankyrin (band 2.1), and the Laemmli method[42] to quantify all the others (band 3; band 4.1; band 4.2; band 4.9; band 5; band 6 and band 7).

DNA analysis

DNA from peripheral blood leukocytes was obtained using conventional methodology.[43] All the exons of the PK-LR gene were analyzed through non-radioactive single-strand conformation polymorphism (SSCP) analysis using previously described primers[15,19] in order to determine the location of the mutation. The product of polymerase chain reaction (PCR) product (6 µL) of the sample is mixed with 6 µL of solution of 95% formamide, 10 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), 0.05% bromophenol blue and 0.05% xylene cyanole, and denatured by heating at 95ºC for 5 min followed by rapid cooling in dry ice. Each sample was applied to a non-denaturing 12.5-15% polyacrylamide gel (polyacrylamide:bis ratio 29:1) containing 90 mM Tris borate buffer. Migration conditions were the following: at 20-25ºC, 130 V/cm overnight. Visualization was accomplished through staining with silver nitrate.

SSCP analysis was followed by a direct *sequencing* of the exon in which fragments with abnormal mobility were observed (indicative of the presence of a mutation), through automatic *sequencing* with the ABI Prism™ dRhodamine Terminator Cycle Sequencing ready reaction Kit (PE Applied Biosystems, Foster City, AC, USA), following the manufacturer's instructions. The sequence reaction was analyzed in an ABI Prism 373A Genetic Analyzer (PE Applied Biosystems).

Family study

Endonuclease restriction digestion (PCR-ER) analysis was performed in all family members. The existence of the mutation was proven by the fact that it destroyed a restriction site for the enzyme *Bst N I*.

Results

Basic hematologic data are summarized in Table 1. Both the propositus and his father showed marked increases in reticulocyte count, MCHC and osmotic fragility test (OFT) using fresh and incubated RBCs. The propositus and his father also had low scores on the acidified glycerol lysis test (AGLT). Careful examination of peripheral RBC morphology in the propositus showed a significant number of spherocytes, ekynocytes and pincered RBCs (Figure 1). The family study demonstrated the presence of spherocytes and pincered cells in the father, and ekynocytes in the mother and brother (Table 1).

RBC membrane protein study demonstrated a significant decrease in band 3 and protein 4.2 content in the propositus and his father (Table 2). Figure 2 is

Table 1. Basic hematologic data, including OFT, AGLT and blood cell morphology.

	Father	Mother	Propositus	Brother	RV
RBC count (10^{12}/L)	3.4	4.56	4.31	4.77	5.4±1.5
Hematocrit (%)	31.6	40	34	39	45±5
Hb (g/L)	110	133	132	135	145±20
MCV (fL)	93	86	78.7	82	88±10
MCH (pg)	32.4	29.1	30.5	28.3	29.5±2.5
MCHC (g/L)	390	334	388	350	325±25
Reticulocytes (%)	4.4	9.0	1.35	0.8	0.2-2
Fresh OFT (g/L)	4.7	4.3	4.7	4.25	4-4.5
Intubated OFT (g/L)	6.4	5	6.15	5	5-5.5
AGLT (s)	<120	>1800	<120	>1800	>1800
Red blood cell morphology					
Spherocytes	++	–	++	–	NP
Pincered cells (%)	0.9%	NP	>0.5%	NP	NP
Ekynocytes (‰)	6	NP	5	4	NP

RBC: Red blood cell. Hb: hemoglobin. MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; OFT: osmotic fragility test. AGLT: acidified glycerol lysis test. RV: reference values (± 2 SD). NP: not present.

a graphic representation of this decrease. RBC enzyme activities studied in the propositus and his family are shown in Table 3. PK activity was low in the propositus and his brother, but normal in both parents.

PCR-SSCP analysis of the PK-LR gene in all the patient's relatives demonstrated a single nucleotide change (1516 G→A) in exon 11 of the propositus and his mother. This change results in substitution of the amino acid residue valine by isoleucine at position 506. In this mutation one *Bst N I* restriction enzyme target is destroyed, leading to the attainment

Table 2. RBC membrane protein data.

	Band 3		Protein 4.2	
	Relative value*	Decrease (%)°	Relative value*	Decrease (%)°
Propositus	21.6	19	3.4	19
Father	22.1	17	3.4	20
Mother	26.8	0	3.8	0
Brother	27.3	0	4.0	0
Control	26.4	0	3.9	0
Ref. values (± 2 SD)	24.8-28.4		3.5-5.0	

**RV: relative value. % total membrane protein.°% of decrease is calculated from the reference mean values (X).*

of only two fragments (186 and 115 bp) instead of the three attained in the normal allele (174, 115 and 12 bp). PCR digestion in exon 11 from the propositus and his mother resulted in restriction fragments of 186, 174, 115 and 12 bp length, indicating a carrier state for the mutation (Figure 3).

Discussion

Hereditary spherocytosis is the most common cause of hereditary anemia in Spain and patients usually present slight to moderate hemolytic anemia, jaundice and splenomegaly. HS is a molecular and clinically heterogeneous disorder mainly due to ankyrin (ANK) deficiency in the red blood cell membrane. In general, its inheritance pattern is autosomal dominant, although autosomal recessive variants have also been reported.[2]

Pyruvate kinase (PK-R) deficiency is another RBC defect due to an enzymopathy of anaerobic glycolysis which results in a clinical picture similar to that of HS.[4] PK-R deficiency is found world-wide, inherited

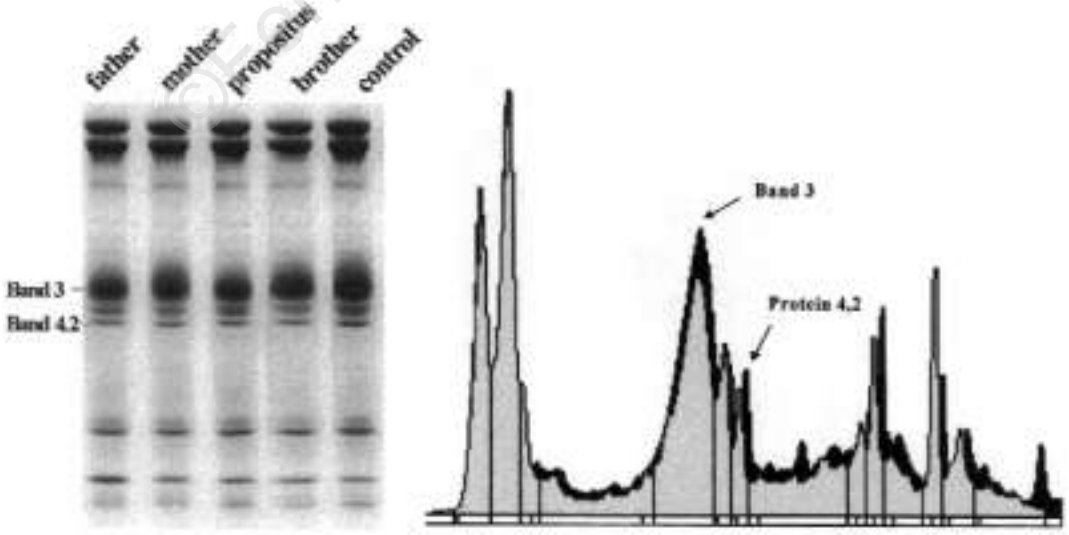

Figure 1. Electrophoretic and densitometric profiles of red cell membrane protein in the patient, his relatives and controls. Electrophoretic profiles are shown on the left, and densitometric profile of the patient in comparison with the control is shown on the right.

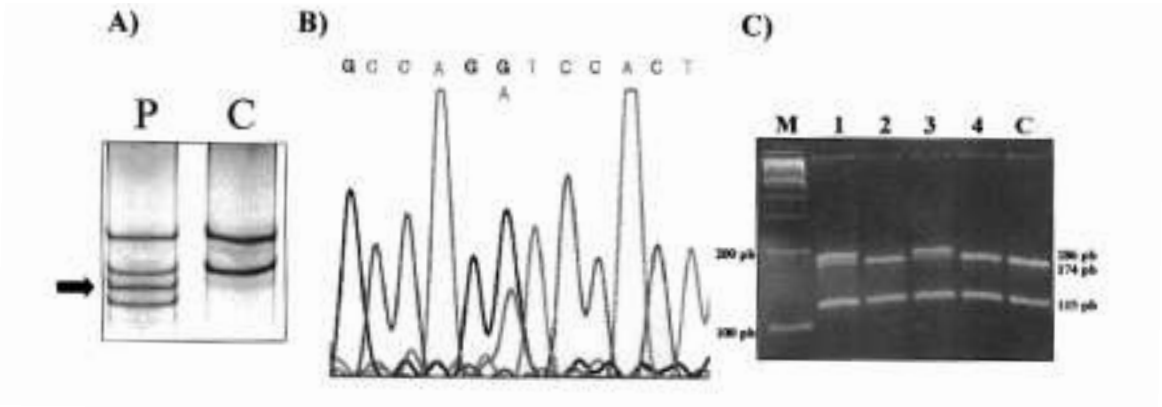

Figure 2. A) SSCP analysis of PCR-amplified DNA fragment containing the exon 11 of PK-LR gene of the patient (P) showed abnormal mobility compared to the control sample (C). B) Detection of the G→A mutation at nucleotide 1516 by automatic sequencing. C) Confirmation of the presence of the mutation with restriction endonuclease digestion, using the Bst NI. M: molecular-wight marker 100 bp DNA ladder; 1: patient; 2: father; 3: mother; 4: brother; C: normal control.

Table 3. RBC enzyme activities.

	Father	*Mother*	*Propositus*	*Brother*	*RV*
G6PD	8.5	7.8	8.8	7.9	5.7-9.9
PK	8.9	10.5	7.3	8.0	8.4-15.2
HK	0.86	0.85	0.87	0.87	0.6-1.26
PK/HK	10.3	12.3	8.39	8.9	7.2-15.6
GPI	63	48	64	50	45-66
AChE	37	30	32	27.1	26-43

Data expressed in IU/g Hb. G-6PD: glucose-6-phosphate dehydrogenase; PK: pyruvate kinase; HK: hexokinase; GPI: glucose phosphate isomerase; AChE: acetylcholinesterase.

in an autosomal recessive mode and clinically characterized by chronic hemolysis of variable severity. Spiculated RBCs are frequently seen on careful examination of peripheral blood. Splenectomy often results in a decrease of hemolytic anemia severity, especially in more seriously affected patients. Heterozygous carriers usually exhibit about 40 to 60% of normal PK activity and the disease is not clinically expressed. Affected patients are usually heterozygous compounds for two mutant alleles, except in families in which consanguinity is present.

Partial PK deficiency has been associated with other hematologic disorders such as G6PD deficiency, α-thalassemia, hereditary elliptocytosis and Gaucher's disease as well as several hematologic malignancies.[11] However, the association of PK deficiency and hereditary spherocytosis is much less frequent than the relatively high prevalence of both disorders might sug-

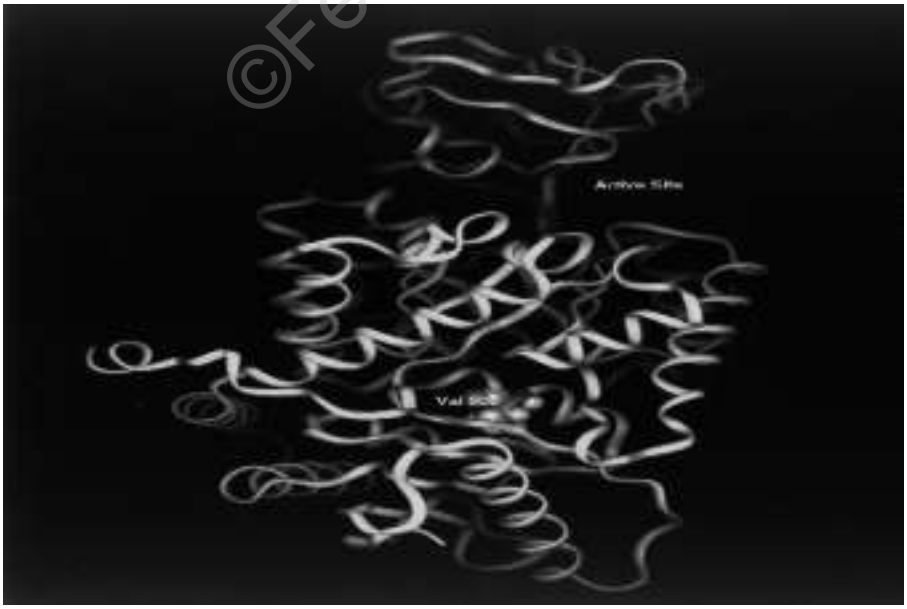

Figure 3. Solid ribbon structure of the one subunit of PK after simulation and energy minimization, using the programs Insight-II (v.2.3.5) and Discover (v. 2.9.7) (Biosym tech., San Diego, CA, USA) on a Silicon Graphics R8000 workstation. The position of the affected residue is indicated.

gest; only one case has been reported, by Brook and Tanaka.[10] We describe here a further case of concomitant HS and PK deficiency in a patient with chronic nonspherocytic hemolytic anemia. Molecular study of the *PK* gene allowed us to identify a new PK variant, provisionally designated as PK Mallorca. Furthermore, the family study demonstrated the hereditary transmission pattern of both RBC disorders and contributed to explaining the clinical behavior observed in each family member: the propositus inherited a RBC membrane defect (band 3 and protein 4.2 deficiency) from his father, who exhibited a moderate chronic hemolytic anemia, and a mutated *PK-R* gene allele from his mother, who was asymptomatic. Despite the presence of an evident HS defect, demonstrated by increased osmotic fragility tests, circulating spherocytes and an abnormal RBC membrane protein pattern, clinical manifestations were less severe in the propositus than in his father. As we reported in a previous paper,[2] band 3 deficiency alone or associated with protein 4.2 deficiency, is the most frequent membrane protein abnormality in HS patients of Spanish extract. In addition this protein defect is frequently associated with an increased number of circulating *pincered* RBCs.[44] In the present study, both the propositus and his mother (heterozygous for PK deficiency), exhibited spiculated RBCs not present in the father (with normal PK gene) and *pincered* RBCs (> 0.5%) were also present in the propositus. Spiculated RBCs are relatively frequent in PK deficiency even in the carrier state or without clinical manifestations, as demonstrated here in the propositus's mother, who, despite a mutated PK gene, exhibited normal RBC PK activity.

The new mutation reported here (1516 G→A) responsible for the PK-R Mallorca causes the substitution of a 506 valine to isoleucine (506Val→Ile) in the C-terminal region of the enzyme. Since the 506 Val is not conserved in species such as cats, rats and yeast,[45] it may be assumed that this amino acid has little impact on enzyme function. This idea is further supported by the fact that 506 Val is located far from the active site (A and B zone) of the molecule, but near the union site of the enzyme allosteric activator 1.6-fructose biphosphate.[46]

Finally, our findings emphasize the importance of systematic determination of the key glycolytic enzyme activities in any chronic hemolytic anemia with moderate or severe hemolysis in patients with an underlying RBC membrane defect. This practice would improve our knowledge of the epidemiologic and genetic characteristics of both disorders, and of the frequency of this association.

Contributions and Acknowledgments

RZ was responsible for PCRs and sequencing analysis, literature revision and writing the manuscript. MM ,RA and MAP carried out the RBC membrane protein studies and contributed to data collection, JG and MM were the clinicians responsible for the patient's clinical management. JLVC supervised the entire study and revised the final version of the paper.

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Disclosures

Conflict of interest: none.

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Potential implications for clinical practice

- Systematic key glycolytic enzyme measurements should be considered in any red cell membrane defect associated with chronic hemolytic anemia.

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