

Coexistence of congenital red cell pyruvate kinase and band 3 deficiency

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Summary The authors report the case of a 9-year-old Caucasian girl, born in northern Portugal, with chronic nonspherocytic haemolytic anaemia and without family history of anaemia. The aetiological study of this anaemia revealed pyruvate kinase deficiency (PKD), because of two previously described mutations (426Arg → Trp and 510Arg → Gln). Since the blood smear revealed features not fully compatible with PKD diagnosis, additional tests were performed for the propositus and her parents, namely red blood cell membrane protein analysis. A decrease in proteins band 3 (15%) and 4.2 (18%) was found in the propositus. Her father presented only a decrease in band 3 (11%). Coexistence of PKD and erythrocyte membrane proteins deficiency in the same patient is very uncommon. Our findings suggest that a careful blood smear observation may lead to the identification of a combined deficiency in erythrocyte membrane proteins and enzymopathies.

Keywords Band 3 deficiency, chronic nonspherocytic haemolytic anaemia, hereditary spherocytosis, pyruvate kinase

Introduction

Pyruvate kinase (PK) deficiency is the most common enzyme abnormality in the erythrocyte glycolytic pathway causing hereditary chronic nonspherocytic haemolytic anaemia. Usually, clinical manifestations are only present in homozygous or compound heterozygous subjects for different gene mutations (Baronciani & Beutler, 1993; Kanno, Fujii & Miwa, 1993; Demina *et al.*, 1998; Ferreira *et al.*, 2000). Considering the heterogeneity of the underlying molecular defects, it is not always possible to make a precise genotype/phenotype correlation. Heterozygote car-

riers of the enzyme deficiency are usually devoid of clinical manifestations (Demina *et al.*, 1998; Ferreira *et al.*, 2000). Red cell morphology is commonly unremarkable, displaying some degree of anisocytosis and polychromatophilia with a variable number of contracted echinocytes (small densely staining spiculated cells), which usually become more numerous after splenectomy (Zanella & Bianchi, 2000).

Band 3, the red cell anion exchanger, is a member of a widely distributed family of proteins present in many tissues, which play part in many cellular processes involving the exchange transport of anions across membranes (Kollert-Jons *et al.*, 1993; Sahr *et al.*, 1994; Schofield *et al.*, 1994). In red cells, band 3 enhances the ability of blood to carry carbon dioxide from the tissues to the lungs, and contributes to the stability and integrity of the cell. Band 3 is the most representative protein in RBC membrane and its deficiency is associated with

Accepted for publication 12 April 2004

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20–40% (Hassoun & Palek, 1996) of all hereditary spherocytosis cases, as well as with a few elliptocytosis cases (namely southeast Asian ovalocytosis) (Jarolim *et al.*, 1991).

We describe a concomitant PK and band 3 deficiency in a female child with chronic haemolytic anaemia.

Case report

The patient is a 9-year-old Caucasian girl born in northern Portugal of nonconsanguineous parents with no known family history of anaemia. She developed jaundice in the first 24 h of life needing no specific treatment, and had a history of chronic anaemia with sporadic iron supplementation.

We first saw the patient when she was 6 years old. She presented normal physical and intellectual development. Her skin was pale, the sclera slightly icteric and the spleen was palpable 2.5 cm below the costal margin.

The basic haematological studies showed anaemia (9.8 g/dl), reticulocytosis ($398 \times 10^9/l$), high levels of total and indirect bilirubin (85 $\mu\text{mol/L}$ and 80.5 $\mu\text{mol/L}$, respectively), slightly high lactate dehydrogenase level

(463 IU/l) and undetectable haptoglobin levels. The blood smear revealed red cell anisocytosis and anisochromia with some elliptical and few tear drop-shaped erythrocytes (Figure 1).

The aetiological study of this haemolytic anaemia revealed a reduced PK activity of 2.2 IU/g Hb [normal range (average \pm 2 SD): 9.35 ± 2.3 IU/g Hb]. The activity of the other erythrocyte glycolytic enzymes, as well as the osmotic fragility test and the haemoglobin electrophoresis pattern were normal, in the patient and in her parents. Both parents presented PK activities in the heterozygous range (father: 3.0 IU/g Hb; mother: 3.2 IU/g Hb). A direct sequencing of *PKLR* gene revealed a compound heterozygosity in the patient for two previously described mutations (Figure 2), 1276T (426Arg \rightarrow Trp) and 1529A (510Arg \rightarrow Gln). Molecular analysis of the TATA-box region of the UDP-glucuronosyltransferase-1 (*UGT1A1*) gene was made, revealing heterozygosity for a TA insertion [(TA)₆/(TA)₇] (Figure 3).

Two years later, her haemoglobin levels dropped further (first year average Hb: 9.15 g/dl; second year average Hb: 8.2 g/dl). At this time, splenectomy was performed. Afterwards, haemoglobin levels showed a rise to stable values above 10 g/dl; reticulocyte count increased to an average of $688 \times 10^9/l$, and total bilirubin levels fell to around 29.2 $\mu\text{mol/L}$. Red blood cell morphology, as expected after splenectomy, acquired slight different features with the presence of some poikilocytes and echinocytes. Few elliptocytes were still seen (Figure 1), which led us to search for membrane protein deficiencies. The erythrocytes were isolated and their membranes obtained and prepared according to Dodge, Mitchell and Hanahan (1963). Protein concentration of the membrane suspensions was determined (Bradford, 1976), and, after heat denaturation, proteins were submitted to polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS-PAGE), according to the methods of Laemmli (1970) and Fairbanks, Steck and Wallach (1971). Proteins were stained with Coomassie blue, and the gels were analysed by a densitometer coupled to an image analysis software (Bio-profil; Vilbert Lourmat, Torcy Marne-la-Vallée, France). We found a 15% decrease of band 3 and an 18% decrease of protein 4.2 in the patient, whereas the father presented only a 11.3% decrease in band 3 (Figure 4), suggesting a primary band 3 deficiency in both.

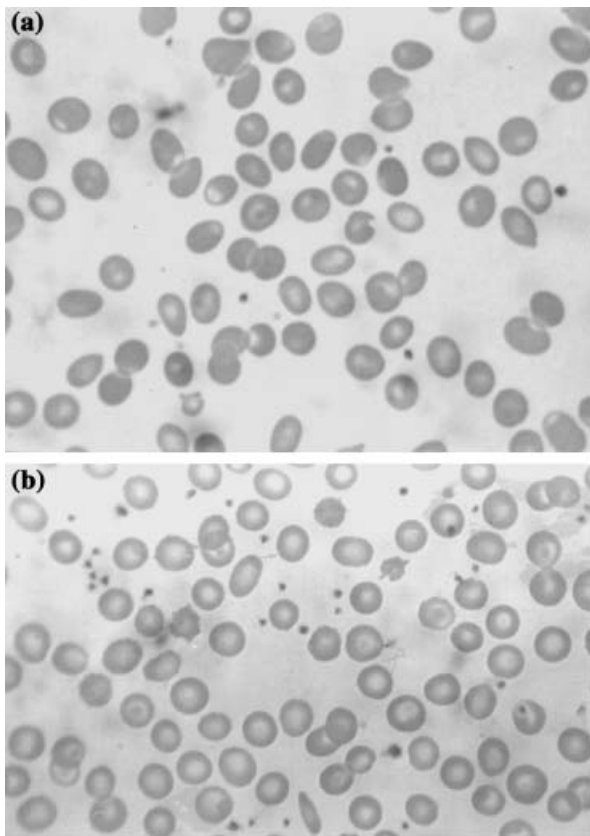


Figure 1. Blood smear of the propositus, before (a) and after (b) splenectomy.

Discussion

Unlike PK deficiency, which is a rare condition, band 3 deficiency is one of the most frequent causes of hereditary

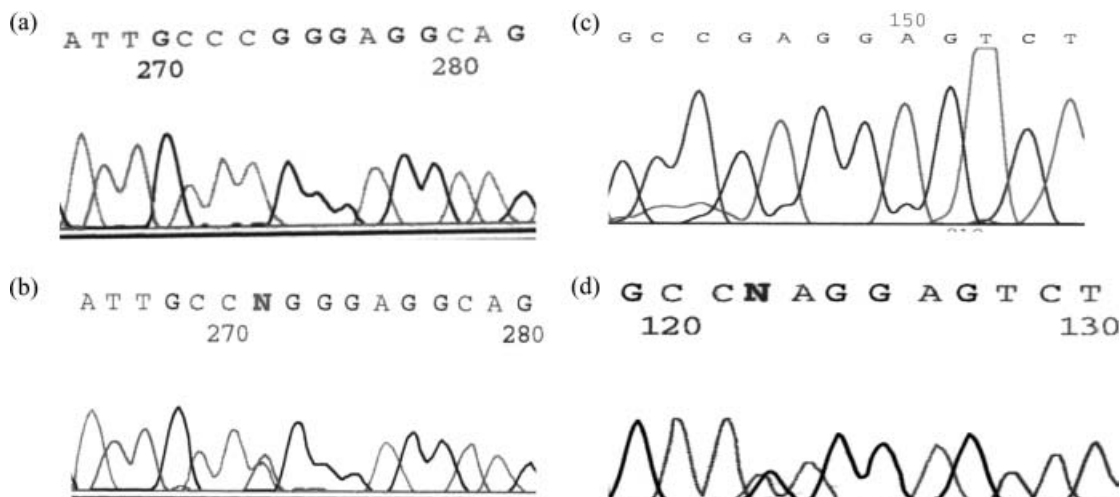


Figure 2. Extract of direct sequencing of exon 10 and 11 of the *PKLR* gene. (a) Normal sequencing of exon 10. (b) Patient sequencing of exon 10, with C and T peaks in nucleotide 1276 (426Arg → Trp). (c) Normal sequencing of exon 11. (d) Patient sequencing of exon 11, with G and A peaks in nucleotide 1529 (510Arg → Gln).

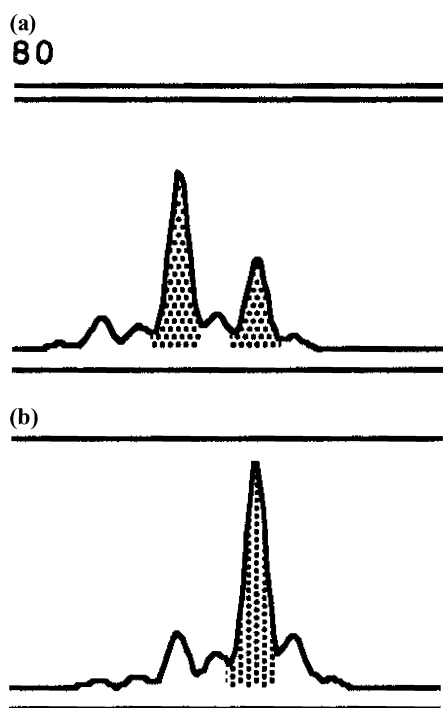


Figure 3. Screening for *UGT1A1* promoter variants, performed by fluorescence-labelled polymerase chain reaction (PCR). The amplified DNA fragment were separated by automated capillary electrophoresis and analysed with ABI GeneScan program (Applied Biosystems, Foster City, CA, USA). (a) Propositus sample heterozygous for the mutant (TA)7 allele. (b) Control sample homozygous for the (TA)7 allele.

haemolytic anaemia. The combination of both is very uncommon. To our knowledge, only one report by Zarza *et al.* (2000) describes the combination of partial band 3

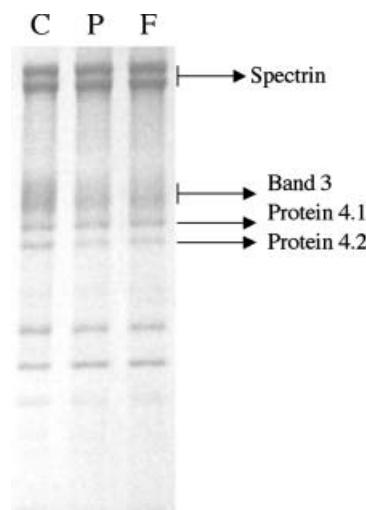


Figure 4. Red cell membrane protein profiles in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels, presented by a control (C), propositus (P) and father (F).

and protein 4.2 deficiency with heterozygosity for a mutation in the *PKLR* gene. In the present case, the patient presented a PK deficiency, with compound heterozygosity for two *PKLR* mutations, combined with band 3 and protein 4.2 deficiency.

In the first approach to the patient, the clinical and, in particular, the laboratorial features were not suggestive of a membrane protein deficiency underlying the chronic haemolytic anaemia presented by the patient. The only feature present in this case, which made us suspect of something more than just PK deficiency, was the observation of elliptocytic cells in the blood smear. Moreover,

the father, who was heterozygous for PK deficiency and who also showed to present a band 3 deficiency, had no remarkable morphological changes in the blood smear. We did not find in the literature detailed descriptions of other cases of compound heterozygosity for the two described PK mutations, to which compare the clinical and blood smear features presented by our patient.

After splenectomy of the propositus, her haemoglobin levels stabilized at a higher level. This was accompanied by an increase in the number of reticulocytes, which is a common feature postsplenectomy in PK deficiency (Zanella & Bianchi, 2000).

The most common genetic variant of *UGT1A1* gene, which is the main cause of Gilbert's Syndrome in the Portuguese population (Costa *et al.*, 2002a), is a TA insertion in the repetitive TATA-box of the gene promoter, which normally consists of six repeats. In the present case, heterozygosity for this TA insertion was also found. This heterozygosity is thought to influence, albeit marginally, the bilirubin levels in patients with chronic haemolytic anaemia (Costa *et al.*, 2002b).

In PK deficiency, genotype/phenotype correlation is difficult to establish. There is a great variability in the possible combinations of the mutant alleles, and, even in patients with the same known genotype (either in homozygosity or compound heterozygosity), a significant variability in phenotype is observed. Coinheritance of erythrocyte membrane protein defects could influence this observed variability. In our case, it is not possible to demonstrate the interference of the present membrane protein defect with the clinical picture shown by the patient. Nevertheless, we believe that a careful blood smear observation could lead to the identification of combined enzyme and membrane protein deficiencies. By doing so, it could be possible to better understand the variability of the phenotypic manifestations and the mutual interference of both deficiencies.

In conclusion, our report emphasizes the importance of a careful blood smear observation in cases of chronic haemolytic anaemia, and the need for additional studies, whenever features do not entirely fit within the initially proposed diagnosis.

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