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Red cell enzyme deficiencies: molecular and clinical aspects

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A B S T R A C T

Over the past few years the inherited disorders of erythrocyte metabolism have been the object of intensive research which has resulted in a better understanding of their molecular basis. Many genes encoding for red cell enzymes of various metabolic pathways have been cloned and numerous mutations identified. More recently, the comparison of the recombinant mutants with the wild-type enzymes has enabled the effects of amino acid replacements on the enzyme molecular properties to be determined and help to correlate genotype to clinical phenotype. However, the clinical manifestations of red cell enzyme defects are not merely dependent on the molecular properties of the mutant protein but rather reflect the complex interactions of additional factors, including genetic background, concomitant functional polymorphisms of other enzymes, posttranslational or epigenetic modifications, ineffective erythropoiesis and differences in splenic function.

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Red cell enzyme deficiencies can lead to different clinical phenotypes: haemolytic anaemia, methemoglobinaemia, and erythrocytosis. Chronic non-spherocytic haemolytic anaemia (CNSHA) is associated with abnormalities of enzymes of the Embden-Myerhof pathway (glycolysis) and nucleotide metabolism, with the exception of the class-I glucose-6-phosphate dehydrogenase (G6PD) variants in the pentose phosphate shunt, which also result in chronic haemolysis and neonatal jaundice. The degree of haemolysis is variable and depends on the metabolic cycle involved, the relative importance of the affected enzyme, and the properties of the mutant enzyme with regard to kinetic abnormalities and/or instability. The ability to compensate for the enzyme deficiency by over-expressing isoenzymes or using alternative pathways contributes to the variability of clinical picture. Moreover, if the defective enzyme is not confined to the red cells but also expressed in other tissues, non-haematological symptoms may occur. Defects of ubiquitous enzymes may cause prenatal mortality and therefore be rarely seen by clinicians. This review summarises the current knowledge in the genetic and clinical features of the most frequent and/or severe enzyme defects of glycolysis and nucleotide metabolism.These enzymopathies are likely to be underestimated, the laboratory diagnosis being sometimes complex and not generally available.

Glycolytic defects associated with CNSHA

Pyruvate kinase (PK) deficiency

Pyruvate kinase is a key glycolytic enzyme that catalyses the transphosphorylation from phosphoenolpyruvate to ADP, yielding pyruvate and ATP. PK deficiency, firstly identified in the early '60s,[1] is the most common cause of CNSHA together with class-I G6PD deficiency. As most red cell enzyme defects, it is transmitted as an autosomal recessive trait: it has a world-wide geographical distribution, with a prevalence of 1:20,000 in the general white population as assessed by gene frequency studies.[2] Clinical manifestations comprise the usual hallmarks of life-long chronic haemolysis. The severity of haemolysis is variable, ranging from mild or fully compensated anaemia to life-threatening neonatal haemolysis necessitating exchange transfusions and subsequent continuous transfusion support.[3] Hydrops foetalis and death in the neonatal period have also been reported in rare cases.[4-9] In infants the anaemia tends to improve with ageing, and may even become fully compensated in some cases. The degree of anaemia is relatively constant in adulthood, although occasional exacerbation may occur during acute infections and pregnancy. It is worth noting that the anaemia may be surprisingly well tolerated in PK deficient patients because of the increased red cell 2,3-DPG content, which results in a decreased haemoglobin's oxygen affinity.[3]

In a series of 61 cases from 54 families referred to our Centre, the median age at the time of diagnosis was 16 years (range 1 day to 65 years): in a quarter of patients the diagnosis was made within the first year of life. Anaemia was severe in 17 and moderate-to-mild in 31 of not splenectomised (or before splenectomy) patients. Six more cases were not anaemic, and the disease was detected in adult age by chance, or in concomitance with pregnancy.

Neonatal jaundice was common, requiring exchange transfusion in 25/33 patients; one case died during exchange transfusion soon after birth. The early onset of symptoms is usually associated with a severe clinical course: 16 out of the 25 exchange-transfused newborns subsequently required multiple transfusions and/or splenectomy. Overall, 65% of patients received blood transfusions (1 to >100, median 15), of whom half were transfusion dependent in childhood or until splenectomy. Gallstones are detected with increased frequency after the first decade of life, and may occur even after splenectomy.

Iron overload is a frequent complication even in not transfused patients.[10,11] It has been shown that the presence of hemochromatosis mutations C282Y and H63D at the heterozygous state[11] may contribute to iron overload in co-operation with other factors such as chronic haemolysis, splenectomy, and ineffective erythropoiesis in some cases.[10]

The haematological features of PK deficiency are common to other hereditary non-spherocytic haemolytic diseases. In our series, median haemoglobin concentration was 9.8 g/dL in not splenectomised patients and 7.3 g/dL in candidates to splenectomy. Splenectomy usually resulted in stabilisation of the haemoglobin to a slightly higher levels (median Hb increase 1.8 g/dL, range 0.4-3.4), and in the reduction or even elimination of transfusion requirement in most transfusion-dependent cases. The reticulocyte number in not splenectomised subjects is usually increased (median $166x10^9$/L); however, reticulocytosis is not proportional to the severity of haemolysis contrary to what observed in other haemolytic diseases, since younger PK defective erythrocytes are known to be selectively sequestered by the spleen.[12] Consequently, splenectomy results in a conspicuous rise of reticulocytes (median $796x10^9$/L), even if the anaemia becomes less severe. Red cell morphology is commonly unremarkable.

Since the haematologic features are not distinctive, the diagnosis ultimately depends upon the determination of enzyme activity, although this can be normal or even increased in some patients.[13] PK activity is neither related to the severity of haemolysis, nor to the reticulocyte number.[3] Care must be taken in interpreting *in vitro* PK assays: contamination with normal donor red cells in transfused patients, incomplete leukocyte removal and compensatory persistence of the M_2 isoform[14,15] may result in a falsely normal red cell enzyme activity. Moreover, kinetically abnormal mutant PKs, although ineffective in vivo, may display normal or even higher catalytic activity under the optimal, artificial conditions of laboratory assay.[13]

The *PK-LR* gene is located on chromosome 1q21 where it directs tissue-specific transcription for both the liver-specific (LPK) and the red cell-specific (RPK) isoenzyme by the use of alternate promoters.[16,17] 186 mutations associated with non spherocytic haemolytic anaemia have been so far reported in the *PK-LR* gene.[3,9,18] The mutations identified are mostly missense (69%), splicing and stop codon (13% and 5% respectively), whereas small deletions, insertions and frameshift mutations are rare (11%). Only a few large deletions have been reported.[8,19,20]

The most prevalent mutations in PK deficiency, 1529A and 1456T, have been found to be distributed with a strong ethnic and regional background: 1529A is common in the USA (42%)[19] and in Northern and Central Europe (41%),[15] whereas 1456T is prevalent in Southern Europe (about 30%).[13,21,22] Other mutations, in particular 721T and 994A, are present with a lower frequency in Caucasians.[8,19,21] Clinical studies[13] indicated that severe syndrome was commonly associated with some homozygous missense mutations (in particular 994A and 1529A), or with disruptive mutations such as stop codon (for example 721T), frameshift and splicing mutations. The rare patients with homozygous *null* mutations displayed intrauterine growth retardation, severe anaemia present at birth, need of exchange transfusion and transfusion dependence until splenectomy and, in rare cases, intrauterine death or death in the first days of life.[7-9,23-26]

More recently, the production and characterisation of ten recombinant mutant proteins of human RPK (*Ala137Thr, Gly332Ser, Gly364Asp, Thr384Met, Asp390Asn, Arg479His, Arg486Trp, Arg504Leu, Arg510Gln, and Arg532Trp*) made it possible to define the effects of amino acid replacements on the stability and kinetic properties of PK and helped to correlate genotype to clinical phenotype.[3,27,28]

1529A mutation (Arg510Gln) at the homozygous state results in very low residual PK activity associated with severe to moderate anaemia (Hb levels 5.8-12.2 g/dL).[13] The recombinant mutant protein[27] shows a kinetic behaviour toward ADP and PEP similar to that of the wild-type enzyme but exhibits an higher susceptibility to ATP inhibition and most of all a dramatically lowered thermal stability. Thus, 1529A PK deficiency appears to be primarily due to a lowered intracellular level of RPK, rather than to the altered kinetic and regulatory properties of the enzyme.

Mutation 1456T (Arg486Trp) changes the local conformation of the protein and the local distribution of the charges. The mutant three-dimensional structure shows that the Trp side chain is accommodated without any structural perturbation. Actually, this mutant is even more stable than the wild-type protein and properly responsive to effectors, the only significant perturbation being in the catalytic efficiency, which drops to 30% in respect to the wild-type. The moderate alterations of the kinetic parameters of Arg486Trp mutant correlate with the clinical symptoms because the few 1456T homozygous patients generally exhibit a lifelong history of mild anaemia (haemoglobin 10-12 g/dL).[29] The mild nature of the anaemia may make this mutation underdiagnosed, and this could explain why 1456T is only rarely found in the homozygous state in spite of being one of the most frequent mutations.[2]

Mutation 994A (Gly332Ser) leads to a significant decrease of the catalytic efficiency and drastic reduction of stability, accounting for the very severe haemolytic anaemia (haemoglobin 4.2-7.4 g/dL, transfusion dependence until splenectomy) displayed by the three homozygous patients reported.[8,13] In one family this defect was associated with intrauterine death.[8]

The molecular characterisation of RPK mutants highlights that mutations affect to a different extent thermostability, catalytic efficiency and regulatory properties. Mutations greatly impairing thermostability and/or activity are associated with severe anaemia, but also mutations causing moderate kinetic alterations may give rise to mild to severe anaemia, underlining the essential role of RPK for the entire erythrocyte metabolism. Although there is, in general, correlation between the nature and location of the replaced amino acid and the type of molecular perturbation, caution is needed in predicting the consequence of a mutation simply considering the target residue *per se*: in fact, the clinical manifestations of a genetic disease reflect the interactions of a variety of physiological and environmental factors and do not solely depend on the molecular properties of the altered molecule. Actually, intra-family variability of clinical pattern has been reported in some PK deficient kindred,[7,15] and related to possible individual differences in metabolic or proteolytic activity that may diversely modulate the basic effect of the mutation, or to the compensatory persistence of the M_2 enzyme.[14,15] Moreover, other factors such as recurrent infections, ineffective erythropoiesis and different splenic functions may interfere with the severity of anaemia. In addition, iron overload may greatly impair the clinical course of the disease.[10,11]

Glucose phosphate isomerase (GPI) and hexokinase (HK) deficiency

After PK, the most common glycolytic enzymopathies associated with CNSHA are glucose phosphate isomerase and hexokinase deficiency (reported in 50 and 17 families world-wide, respectively). *GPI* catalyses the interconversion of G6P into F6P in the second step of the Embden-Meyerhof pathway, but it also exerts cytokine properties outside the cells and is involved in several extracellular processes.[30] Twenty-nine mutations have been detected in *GPI* gene, mostly characterised by thermolability with almost normal kinetic properties.

Hexokinase catalyses the phosphorylation of glucose to G6P and is one of the rate limiting enzymes of glycolysis together with PK and phosphofructokinase.

Both GPI and HK deficiency results in haemolysis of variable degree, from severe neonatal hemolysis requiring exchange transfusion to a fully compensated chronic haemolytic anaemia. In rare cases, GPI deficiency also affects non erythroid tissues, causing neurologic symptoms and granulocytes dysfunction.[31] Hydrops foetalis appears to be more common in GPI deficiency than in other enzymopathies. Intrauterine death has been described in one patient carrying an out-of-frame lethal homozygous deletion of *HK1* gene.[32]

Glycolytic defects associated with CNSHA and non-haematological symptoms

Triose phosphate isomerase (TPI) deficiency

Human triose phosphate isomerase is a homodimeric enzyme that catalyses the interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (DHAP) and is involved in glycolysis as well as in gluconeogenesis and triglyceride synthesis.[33] The enzyme is expressed in all cell types and is encoded by a single gene (*TPI1*) at locus 12p13.[34]

TPI deficiency, an autosomal recessive disorder, has been known since 1965.[35] The clinical syndrome in homozygotes or compound heterozygotes is characterised by haemolytic anaemia at onset, often accompanied by neonatal hyperbilirubinemia requiring exchange transfusion.[36] In addition, all the described patients but two[37,38] display progressive neurologic dysfunction, increased susceptibility to infection, and cardiomyopathy.[36] Neurologic complications, most often beginning in the first months of life, include dystonia, tremor, pyramidal tract signs, and spinal motor neuron involvement.[39] Patients show a 20- to 60-fold increased DHAP concentration in their erythrocytes, consistent with a metabolic block at the TPI step. There is no specific treatment, but aggressive supportive care, especially assisted respiration, has appeared to prolong life in some instances. Most affected individuals die in childhood before the age of 6 years, with some exceptions. An Hungarian family has been described, in which 2 adult germline-identical compound heterozygous brothers (Phe240Leu/Glu145stop) display strikingly different phenotypes: both have

severe decrease in TPI activity and congenital haemolytic anaemia, but the elder brother is free of neurological manifestations, and even the propositus with neurological symptoms is over 20 years old. Studies aimed at the pathogenesis of this differing phenotype point to functional differences in lipid environment influencing the enzyme activities involved in these phenotypic differences.[40,41]

Among the fourteen mutations identified in the human TPI locus,[36] the commonest described (74%) is a point mutation at codon 104 changing Glu to Asp.[42] Knowledge of the crystal structure of the human enzyme has provided insight into the probable effect of this mutation, which likely perturbs the local structure of the active site.

It is noteworthy that there have been no reports of TPI deficiency null alleles occurring as homozygotes or as compound heterozygotes. It is likely that such combinations would be incompatible with life, as is apparently the case with TPI null homozygosity in mouse embryos.[43] Moreover, a surprisingly high incidence of TPI heterozygosity was reported (1 to 5 per 1000) in population studies,[44] and it was suggested that the rarity of affected patients was the result of the embryolethality of the majority of mutations.

Phosphofructokinase (PFK) deficiency

Phosphofructokinase catalyses the rate-limiting phosphorylation of F6P to FBP. Three different subunits have been identified in humans: PFK-M (muscle), PFK-L (liver), and PFK-P (platelet),[45] expressed in a tissue-specific manner. In erythrocytes, 5 isoenzymes of varying subunit composition (M_4, M_3L_1, M_2L_2, ML_3, and L_4) are present.[46]

PFK deficiency is a rare autosomal recessively inherited disorder. Mutations concerning the L subunit will render red blood cells that contain only M_4; patients display a partial PFK deficiency with mild hemolysis and no myopathy. Likewise, a deficiency of the M subunit results in the absence of muscle PFK causing myopathy and a mild haemolytic disorder. Erythrocytes containing only L_4 PFK show lowered 2,3 DPG levels leading to a favourable shift of haemoglobin's oxigen-affinity curve, thereby accounting from the very mild anaemia or even mild erythrocytosis despite shortened erythrocyte survival.

To date, 15 PFK-deficient PFKM alleles from more than 30 families have been characterised.[47]

Phosphoglycerate kinase (PGK) deficiency

Phosphoglycerate kinase generates one molecule of ATP by catalysing the reversible conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate. PGK deficiency is an X-linked disorder characterised by chronic haemolytic anaemia (often fully compensated), dysfunction of the central nervous system, and myopathy. However, the phenotype of PGK deficiency is highly variable because patients usually do not display all 3 clinical features.[48] PGK activity varies between 0% and 20% of normal but there is no correlation of residual enzymatic activity with clinical severity. Sixteen different mutations in *PGK1* gene, mostly missense, have been described in association with PGK deficiency.[47,49]

Defects of nucleotide metabolism

Pyrimidine 5′ nucleotidase deficiency

Inherited pyrimidine 5'-nucleotidase type-I (P5'N-1) deficiency, transmitted as an autosomal recessive trait, is thought to be the most common cause of CNSHA after G6PD and PK deficiency.[50] More than 60 patients have been reported world-wide,[50-56] with presumably large numbers undetected.[51] The prevalence of P5'N-1 deficiency is unknown.

In the 64 patients reported in the literature the median age at diagnosis was 15 years (range 3 months to 64 years). The anaemia was usually mild to moderate, or even fully compensated in rare cases.[55] Severe anaemia occurred in 12% of patients. The degree of anaemia is relatively constant during life, although occasional exacerbation may occur during acute infections and pregnancy, requiring blood transfusions.[57] Jaundice and splenomegaly are common. Half of the patients were splenectomised, most of them before diagnosis, and one third developed cholelithiasis. Overall, 17 patients needed blood transfusions, and three became transfusion dependent.[58-60] Exchange transfusion was required in one case only.

In 7 patients delayed development and learning difficulties of variable degree have been reported.[52,61-63] Database searches have shown the presence of P5'N-1 cDNA libraries from brain tissue;[51] however, the significance of the association between mental retardation and P5'N deficiency is hard to assess; possible confounding factors include kernicterus or other genetic defects in consanguineous families.

The median haemoglobin concentration was 9.5 g/dL (range 2.8-15.2 g/dL). In 7 patients examined before and after splenectomy, surgery resulted in rise of the haemoglobin (median increase 3.2 g/dL, range 0.5-5.2). The blood film is mainly characterised by red cells basophilic stippling (2-12% of erythrocytes)[51,53] due to intracellular precipitation of undegraded RNA;[64] 5-10% of spherocytes, mostly spiculated, were detected in some cases.[53,62,65] Red cell osmotic fragility is usually normal with few exceptions.[57]

Iron status parameters have been investigated in 8 non transfused P5'N deficient patients. Three of them, all splenectomised, displayed overt iron overload and needed iron chelation (HFE profile: wt/wt; C282Y/C282Y; H63D/wt). Iron deficiency secondary to

intravascular haemolysis was reported in one case.[64]

The clinical and haematological features of P5'N deficiency are not distinctive, although the blood film gives a strong clue as to the diagnosis when marked red cell basophilic stippling is seen. Basophilic stippling is a constant but not specific finding in this disease, occasionally occurring in other conditions such as β-thalassemia trait, some haemoglobin variants, sideroblastic anaemia, or lead poisoning. The diagnosis is therefore based on the demonstration of high concentrations of pyrimidine nucleotides and a reduced P5'N-1 activity in red blood cells.

The nucleotides of normal erythrocytes consist largely of purine derivatives (with an absorption maximum at 260 nm), with very low levels of pyrimidine nucleotides (absorption at 280 nm). In P5'N-1 deficiency the accumulation of high levels of pyrimidine nucleotides results in a decrease in the OD260/OD280 adsorbance ratio.[66]

The different procedures for measuring P5'N-1 activity include spectrophotometric determination of the phosphate produced in dephosphorylating reactions,[67] measurement of radioactive nucleoside liberated from a labelled substrate,[68] quantification at 254 nm of nucleosides separated by HPLC,[69] or by micellar electrokinetic chromatography.[70]

Residual P5'N-1 activity is neither correlated with the degree of the haemolysis, nor with the reticulocyte number,[57] supporting the hypothesis that pyrimidine accumulation and metabolic impairment principally occur in the younger erythrocytes of P5'N-deficient patients.[58]

The gene encoding P5'N-1 (*NT5C3, UMPH1*) is located on chromosome 7p15-p14 and splitted into eleven exons (1, 2, R, 3-10) leading to three mRNA forms by alternative splicing of exons 2 and R.[51,55] Twenty different mutations in the *P5'N-1* gene have been identified in 37 patients from 30 families: six are missense, two lead to in-frame aminoacidic deletion, the remaining twelve include nonsense mutations, deletions, insertions, or splice sites alterations, and are expected to generate truncated or aberrant forms of the polypeptide. All the patients described but two are homozygous.

The molecular bases of P5'N-1 deficiency have been recently investigated studying the biochemical properties of four pathological variants (Asp87Val, Leu131Pro, Asn179Ser, and Gly230Arg), obtained by site-directed mutagenesis technology.[71] All the investigated mutant proteins display a reduction of catalytic efficiency, and/or alterations of heat stability, and are therefore expected to lead to a decay of intracellular P5'N-1 activity. This is the case for Asp87Val and Asn179Ser, with patients exhibiting a very low nucleotidase residual activity (2-5%). On the contrary, Leu131Pro and Gly230Arg seem to allow red cells to maintain a rather high activity (up to about 60%), despite the substantial changes in the kinetic and thermostability parameters of mutant enzymes. In the absence of other evidence, this suggests that in some cases P5'N-1 deficiency could be compensated, possibly by other nucleotidases or by alternative pathways in nucleotide metabolism.

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