Disorders of Erythropoiesis

Molecular characterization of five Portuguese patients with pyrimidine 5'-nucleotidase deficient hemolytic anemia showing three new P5'N-I mutations

Four different gene mutations were identified in five unrelated Portuguese patients with pyrimidine 5'-nucleotidase type I (P5'N-I) deficient chronic hemolytic anemia. Mutations 502G→C (168 Gly→Arg), 773T→C (258Ile→Thr) and the insertion of an Alu element in exon 9, leading to skipping of this exon in the mRNA transcript, are newly described mutations whereas mutation 425T→C (142Leu→Pro) has been previously reported.

haematologica 2006; 91:266-267
(*http://www.haematologica.org/journal/2006/02/266.html*)

Pyrimidine 5'-nucleotidase type-I isoform (P5'N-I) is essential for the catabolism of pyrimidine nucleotides. The enzyme preferentially hydrolyzes uridine monophosphate (UMP) and cytidine monophosphate (CMP), which result from RNA degradation during reticulocyte maturation.[1] P5'N-I deficiency (OMIM*606224) causes chronic non-spherocytic hemolytic anemia associated with accumulation of pyrimidine nucleotides within erythrocytes, and marked basophilic stippling on peripheral blood smears.[1] The specific diagnosis depends on demonstration of high concentrations of pyrimidine nucleotides in erythrocytes and severe reduction in P5'N-I activity.[1,2] The P5'N-I gene (*NT5C3*) is localized on chromosome 7p15, and was first reported to have ten exons coding for two alternatively spliced mRNA forms.[3] Recently, a third spliced form was identified, raising the total number of exons to 11 (1-2-R-3 to 10).[4] Molecular characterization of affected patients has so far identified 15 different P5'N-I gene mutations, most of them within exons 8 and 9.[3-7]

In this report we present the molecular characterization of five patients of Portuguese ancestry with P5'N-I deficient chronic hemolytic anemia (Table 1). The diagnosis was based on clinical data, marked basophilic stippling, anemia with increased number of reticulocytes, a decreased purine-pyrimidine nucleotide ratio (<2.29), and demonstration of the absence of functional P5'N-I protein in hemolysates by electrophoretic analysis (Figure 1A). The age of these patients at diagnosis ranged from 3 to 74 years. All patients are the result of known consanguineous marriages, or have ancestries from neighboring villages; we could not establish any relationship between the five patients. Routine hematologic parameters were determined by standard procedures.[8] The purine-pyrimidine nucleotide ratio was calculated using previously published methods.[8] Electrophoretic analysis was performed as reported elsewhere.[9]

After informed consent, peripheral blood was collected from patients, families and unrelated healthy individuals from central Portugal. Genomic DNA was extracted using standard methodologies. The entire coding sequence and regions adjacent to the P5'N-I gene were amplified by polymerase chain reaction using specific oligonucleotides.[5] Exons 7, 8 and 9 were sequenced with the automatic genetic analyzer ABI-Prism 310. The remaining exons were screened by single strand confor-

Table 1. Hematologic, biochemical and molecular data of five Portuguese patients with P5'N-I deficiency.

Patient	Sex	Age (Yr)	Hb (g/L)	Pur:Pyr (OD_{260}/OD_{280})	Retics (% of RBC)	P5'N-I mutation	Effect
1	M	21	125	1.20	10	502G→C	168Gly→Arg
2	M	18	114	1.20	10	773T→C	258Ile→Thr
3	M	77	88	1.34	7	Alu ins	Exon 9 skipping
4	M	5	106	1.20	10	Alu ins	Exon 9 skipping
5	M	53	114	1.08	25	425T→C	142Leu→Pro

Hb: hemoglobin (normal values: adult males 135-170 g/L; 2-9 years 115-145 g/L); Pur:Pyr: purine-pyrimidine nucleotide ratio (normal values = 2.29 or higher); Retics – reticulocytes (normal value < 2% of RBC).

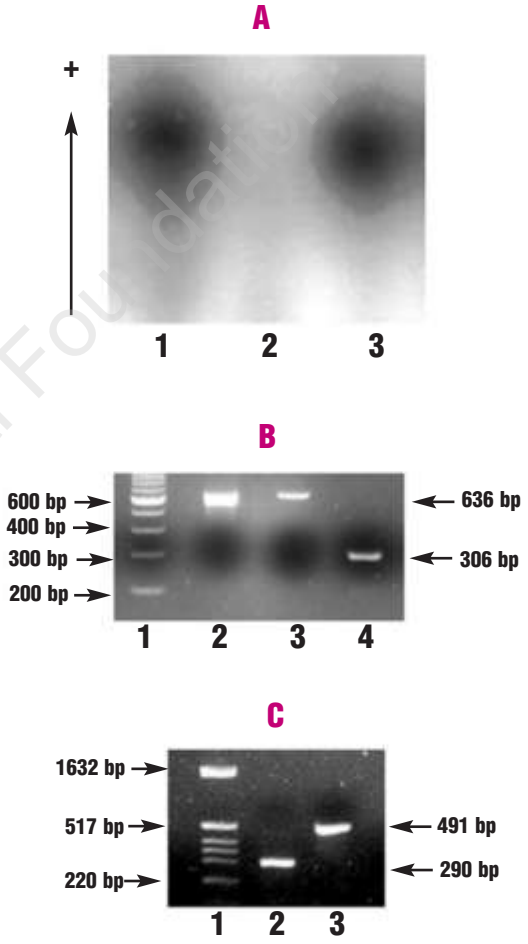

Figure 1. A. Analysis of P5'N-I activity after starch gel electrophoresis of fresh hemolysates from two controls and P5'N-I deficient patient n. 5. The substrate used was UMP. All the five Portuguese patients showed the same electrophoretic pattern as patient n. 5. Lanes 1 and 3 – normal subjects; lane 2 – patient n. 5. **B.** Genomic DNA amplification of P5'N-I exon 9. Agarose gel electrophoresis shows polymerase chain reaction fragments with 636 bp in patients n. 3 and 4 instead of the normal 306 bp fragment. Lane 1, DNA size markers (100 bp ladder); lane 2, patient n. 3; lane 3, patient n. 4; lane 4, normal subject. **C.** Reverse transcription polymerase chain reaction (RT-PCR) analysis of the insertional mutation. To evaluate the effect of the P5'N-I Alu insertion we used the RT-PCR method to generate fragments spanning exons 8 to 10. Agarose gel electrophoresis of RT-PCR products from patient n. 3 shows a faster moving band (290 bp) relative to that of normal controls (491 bp). Lane 1, DNA size markers (pBR322 digested with HinfI); lane 2 - patient n. 3; lane 3 - normal subject.

mational polymerase analysis. Poly(A) mRNA was isolated from reticulocytes of patient 3 and from two control individuals, using the MicroPoly(A)Pure Kit (Ambion). Purified mRNA (10-50 μg) was reverse transcribed (RT) into cDNA using the First-Strand cDNA Synthesis Kit (Amersham Biosciences); a fragment spanning exons 8 to 10 [oligonucleotides: 5'-aagttggtgtccaatttatg-3' (forward) and 5'-gtcaatgcattcaccatatc-3' (reverse)] was amplified and sequenced.

Patient n. 1 showed a previously undescribed mutation in exon 8, the transversion 502G→C, predicting the amino acid change 168Gly→Arg. This is a drastic non-conservative substitution of a flexible amino acid (glycine) by a polar positive residue with a large dissimilar side chain (arginine), increasing the overall rigidity of the polypeptide in this specific region. Gly168 is conserved from *C. elegans* to humans, and falls in a highly conserved region of 7 residues,[3] an area likely to be of functional and/or structural importance

Patient n. 2 has a new mutation in exon 9, the transition 773T→C, predicting the replacement of the aliphatic non-polar amino acid isoleucine, in position 258, by the uncharged polar threonine (258Ile→Thr). The evolutionary conservation of Ile258 from *D. melanogaster* to humans[3] may indicate that this residue has important functional and/or structural features.

In patients n. 3 and 4 amplification of exon 9 (Figure 1B) and sequencing revealed the insertion of a 330 bp Alu repetitive element between nucleotides 743-744. The P5'N Alu element is closely related to the AluYa5 and AluYa1 subfamilies (98% identity). Sequence analysis of reverse-transcriped polymerase chain reaction products from patient n. 3 spanning exons 8 to 10 (Figure 1C) showed a normal nucleotide sequence corresponding to exons 8 and 10, lacking the in-between exon 9. Predicted translation of the abnormal mRNA results in a polypeptide lacking the 67 amino acids encoded by exon 9, which certainly affects the P5'N-I enzymatic activity. It is the first time that a P5'N chronic hemolytic anemia has been associated with an Alu insertion.

Patient n. 5 was found to have a missense mutation in exon 7, 425T→C (142Leu→Pro), which has been previously reported in a Japanese patient[4] Chiarelli *et al.*,[10] studying the biochemical properties of the recombinant protein, showed that it was extremely unstable and suggested that the L142P substitution was the cause of the polypeptide's increased susceptibility to proteolysis.

The three point mutations 502G→C, 773T→C and 425T→C were confirmed by restriction enzyme analysis with *Bst*UI, *Bse*NI and *Sml*I, respectively. No other mutations were found in the remaining P5'N-I gene coding regions or exon/intron junctions, and screening of 60 normal control individuals was negative for the new P5'N-I mutations. All the patients have their mutations in the homozygous state, in concordance with the genetic profile for the disease in other populations.

Licínio Manco,° Luís Relvas,* C. Silva Pinto,* Janet Pereira,* A. Bessa Almeida,# M. Letícia Ribeiro**

**Unidade de Hematologia Molecular, Centro Hospitalar de Coimbra, Portugal; °Departamento de Antropologia, Universidade de Coimbra, Portugal; #Serviço de Hematologia, Hospital D. Estefânia, Lisboa, Portugal*

Funding: this work was performed in part with the support of Centro de Investigação em Antropologia, Universidade de Coimbra and Forum Hematológico, Serviço de Hematologia, Centro Hospitalar de Coimbra.

Key words: pyrimidine 5'-nucleotidase deficiency, hemolytic anemia, P5'N-I gene, Alu insertion, RT-PCR analysis.

Correspondence: Maria Letícia Ribeiro, MD, PhD, Unidade de Hematologia Molecular, Hospital Pediátrico, Centro Hospitalar de Coimbra, 3000 Coimbra, Portugal. Phone: international +351.239480347. Fax: international +351.239717216. E-mail: leticia.ribeiro@hpc.chc.min-saude.pt

References

1. Valentine WK, Fink K, Paglia DE, Harris SE, Adama WS. Hereditary hemolytic anemia with human erythrocyte pyrimidine 5'-nucleotidase deficiency. J Clin Invest 1974;54:866-79.
2. Rees DC, Duley JA, Marinaki AM. Pyrimidine 5' nucleotidase deficiency. Br J Haematol 2003;120:375-83.
3. Marinaki AM, Escuredo E, Duley JA, Simmonds HA, Amici A, Naponelli V, et al. Genetic basis of hemolytic anemia caused by pyrimidine 5' nucleotidase deficiency. Blood 2001;97:3327-32.
4. Kanno H, Takizawa T, Miwa S, Fujii H. Molecular basis of Japanese variants of pyrimidine 5'-nucleotidase deficiency. Br J Haematol 2004;126:265-71.
5. Balta G, Gumruk F, Akarsu N, Gurgey A, Altay C. Molecular characterization of Turkish patients with pyrimidine 5' nucleotidase-I deficiency. Blood 2003;102:1900-3.
6. Bianchi P, Fermo E, Alfinito F, Vercellati C, Baserga M, Ferraro F, et al. Molecular characterization of six unrelated Italian patients affected by pyrimidine 5'-nucleotidase deficiency. Br J Haematol 2003;122:847-51.
7. Escuredo E, Marinaki AM, Duley JA, Thein SL, Rees C. The genetic basis of the interaction between pyrimidine 5' nucleotidase I deficiency and hemoblobin E. Nucleosides Nucleotides Nucleic Acids 2004;23:1261-3.
8. Dacie JV, Lewis SM. Practical Haematology. 9th edition. Churchill Livingstone, London. 2001.
9. Manco L, Amorim A. Human erythrocyte pyrimidine 5'-nucleotidase isoenzymes: effect of sulfhydryl reagents and electrophoretic discrimination. Electrophoresis 1993;14:1084-5.
10. Chiarelli LR, Bianchi P, Fermo E, Galizzi A, Iadarola P, Mattevi A, et al. Functional analysis of pyrimidine 5'-nucleotidase mutants causing nonspherocytic hemolytic anemia. Blood 2005;105:3340-5.