Two new mutations of the P5’N-1 gene found in Italian patients with hereditary hemolytic anemia: the molecular basis of the red cell enzyme disorder

Laurent R. Chiarelli
Elisa Fermo
Patrizia Abrusci
Paola Bianchi
Chiara M. Dellacasa
Alessandro Galizzi
Alberto Zanella
Giovanna Valentini

Inherited pyrimidine 5’-nucleotidase type-1 (P5’N-1) deficiency is the most frequent abnormality of red cell nucleotide metabolism causing non-spherocytic hemolytic anemia. We describe two novel mutations in two Italian patients affected by P5’N-1 deficiency. One mutation is a two base deletion that occurs at the splice site junction between intron 7 and exon 8 (c.396-397del AG); the second is an in-frame deletion of three adjacent bases (c.427-429del CAA), leading to deletion of glutamine 143. The kinetic properties of Q143del variant were not grossly altered, but the variant was very heat unstable even at physiological temperatures.

Key words: P5’N-1 deficiency, hemolytic anemia, mutations, recombinant mutant.

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Design and Methods

Patients
Case 1. The propositus was a 37-year-old woman originating from southern Italy who had been affected by moderate chronic hemolytic anemia since childhood. She also had jaundice, hepatomegaly and gallstones,
but no splenomegaly. The patient needed one blood transfusion during a pregnancy.

Case 2. The propositus, a 37-year-old woman originating from Northern Italy, had a history of chronic hemolytic anemia since childhood with jaundice, splenomegaly, and hepatomegaly. She was found to be a double heterozygote for HbD Punjab and Hb Zurich, which did not, however, justify the degree of hemolysis. Bone marrow morphology showed mild dyserythropoiesis; ferrokinetic studies revealed a slightly increased iron turnover associated with increased peripheral hemolysis. At the age of 14 years she had undergone cholecystectomy and splenectomy, without any significant increase in hemoglobin values. Before splenectomy she had had low serum iron levels (55 µg/dL). Thereafter, progressive rises in serum ferritin and transferrin saturation were observed associated at the age of 22 with marked hepatic siderosis at liver biopsy and increased cardiac iron burden detected by magnetic resonance imaging. The patient was treated with desferrioxamine. At the age of 34 blood transfusions (4U) were required because of exacerbation of anemia (Hb 4.7 g/dL) during parvovirus B19 infection.

Hematologic assays, enzyme detection and molecular studies
Hematologic investigations were carried out as reported elsewhere. The ratio of purine/pyrimidine nucleotides was calculated as described by Beutler et al. P5’N-1 activity was determined by micellar electrokinetic chromatography. After obtaining informed consent, genomic DNA was extracted from leukocytes. The entire coding region and intronic flanking regions of the P5’N-1 gene were analyzed as described previously.

Table 1. Hematologic data of the two P5’N deficient patients at the time of the study.

<table>
<thead>
<tr>
<th></th>
<th>Case 1</th>
<th>Case 2</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>8.4</td>
<td>9.3</td>
<td>11.8-14.7</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>106</td>
<td>124</td>
<td>82-99</td>
</tr>
<tr>
<td>Reticulocytes (×10³/µL)</td>
<td>300</td>
<td>752</td>
<td>24-84</td>
</tr>
<tr>
<td>Unconjugated bilirubin (µM)</td>
<td>54.4</td>
<td>226</td>
<td>&lt;12.8</td>
</tr>
<tr>
<td>Lactate dehydrogenase (µM)</td>
<td>1218</td>
<td>440</td>
<td>230-460</td>
</tr>
<tr>
<td>Serum ferritin (µg/L)</td>
<td>36</td>
<td>1980</td>
<td>19-238</td>
</tr>
<tr>
<td>Transferin saturation (%)</td>
<td>45</td>
<td>100</td>
<td>16-54</td>
</tr>
<tr>
<td>Osmotic fragility</td>
<td>normal</td>
<td>normal</td>
<td></td>
</tr>
</tbody>
</table>

Construction of pEA9 coding for the Q143del mutant form.
The DNA sequence encoding the mutant enzyme Q143del was obtained by a two-step total gene synthesis method. pEA1, a derivative of PET28a(+), containing the P5’N-1 cDNA, was used as the template. The DNA sequence (cttacagAGAAGGAT) is not possible to determine whether the deletion affects the last bases of intron 7 or the first bases of exon 8. In any case, the deletion may affect the splice site resulting in a splicing alteration and in the absence of a functional protein in RBC. Moreover, if splicing should occur, the two bp deletion would generate a frameshift and a premature stop codon at position 135. A functional protein is not expected in this situation either. In fact, P5’N-1 activity could not be detected in the patient’s RBC, despite using a very sensitive detection method such as capillary electrophoresis. Intriguingly, the degree of anemia was moderate, suggesting that there is a mechanism of compensation, as there is in other enzyme deficiencies.

Expression, purification and characterization of the Q143del enzyme
The mutant protein was expressed in E. coli BL21(DE3)pLysS transformed with pEA9 by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (21°C, 12 hours) and purified using the method previously reported. Kinetic analyses, thermal stability assays and far-UV circular dichroism (far-UV CD) measurements were performed as described in the same report.

Results and Discussion
P5’N-1 deficiency (OMIM 606224) has been suggested to be the third most common hereditary red cell enzyme disorder causing hemolysis after glucose 6-phosphate dehydrogenase and pyruvate kinase deficiencies. It can also be acquired as a result of lead poisoning or oxidative threat. In this study, we investigated two Italian patients affected by non-spherocytic hemolytic anemia who were found to have two new mutations in the gene encoding P5’N-1.

The hematologic data at the time of the study are reported in Table 1. In case 1 the molecular analysis of the P5’N-1 gene showed the presence of a new two bp homozygous deletion (c.396-397del AG); given the DNA sequence (cttacagAGAAGGAT), it is not possible to determine whether the deletion affects the last bases of intron 7 or the first bases of exon 8. In any case, the deletion may affect the splice site resulting in a splicing alteration and in the absence of a functional protein in RBC. Moreover, if splicing should occur, the two bp deletion would generate a frameshift and a premature stop codon at position 135. A functional protein is not expected in this situation either. In fact, P5’N-1 activity could not be detected in the patient’s RBC, despite using a very sensitive detection method such as capillary electrophoresis. Intriguingly, the degree of anemia was moderate, suggesting that there is a mechanism of compensation, as there is in other enzyme deficiencies.

In case 2 complete sequencing of the P5’N-1 gene showed that the patient was a compound heterozygote. One allele had the frameshift mutation c.710-711insGG, already described in Italian and Turkish patients, while the second allele showed a new in-frame deletion of three base pairs (c.427-429del CAA), leading to dele-
tion of Q143. The c.710-711insGG mutation is predicted to lead to a truncated enzyme lacking 42 residues at the C-terminus. Thus, conceivably, the gene product is completely unable to display any activity. To evaluate the effect of Q143del on the activity of P5′N-1, the mutant enzyme was produced in *E. coli* as a recombinant form, purified to homogeneity and characterized. Overall, the Q143del mutant enzyme behaved as the wild-type enzyme, exhibiting similar kinetic parameters towards UMP and CMP (Table 2). Only a small decrease (2.5 fold) of the specificity constant (\( k_{cat}/K_m \)) for UMP was observed (86 vs 213 s\(^{-1}\)mM\(^{-1}\)), due to an increase of the \( K_m \) value. Thus, the Q143 deletion turned out to have only marginal effects on the catalytic properties of the enzyme, strongly suggesting that it is not involved in the catalytic site, as supported by structural data.

To unravel the cause of the lowered enzyme activity, the stability of the mutant form was investigated by thermal inactivation assays and circular dichroism analysis. Treated at 46°C, the enzyme showed high thermal instability with the loss of 50% of its initial activity within the first 3 minutes of incubation (Table 2). Moreover, even at 37°C the mutant enzyme was very unstable, with its activity halved within 38 minutes of incubation (Table 2). The evaluation of the behavior of the Q143del mutant in a wider range of temperatures (25°C-60°C) led to a better appreciation of the reduced stability of the mutant with respect to the wild-type enzyme (Figure 1A). The \( T_{50} \) of the mutant enzyme was 8°C lower than that of the wild-type protein (Table 2). The marked instability of the Q143del mutant enzyme is also supported by the findings of the far-UV circular dichroism analysis carried out at 25°C and 37°C (Figure 1B). Compared to the wild type enzyme, Q143del displayed altered \( \alpha \)-helical and \( \beta \)-sheet content (15%±2% and 24%±1% vs 37%±2% and 26%±1% for the wild type) even at 25°C. A substantial drop of the \( \alpha \)-helical content was observed when the temperature was increased to 37°C (3.2%±0.2% and 22%±1% for \( \alpha \)-helices and \( \beta \)-sheets, respectively). Therefore, the deletion of Q143 destabilizes the secondary structure of the enzyme which leads to premature intracellular degradation in vivo. The mutation is similar to the pathological variant L131P, which is also func-

| Table 2. The main features of recombinant Q143del mutant P5′N-1. |
|---|---|---|---|
| enzyme | kinetics | thermal stability |
| | | | |
| CMP | UMP | \( k_{cat}/K_m \) (s\(^{-1}\)mM\(^{-1}\)) | \( k_{cat}/K_m \) (s\(^{-1}\)mM\(^{-1}\)) | \( t_{0.5} \) 37°C (min) | \( t_{0.5} \) 46°C (min) | \( T_{50} \) (°C) |
| wild type | 26.1±0.7 | 145 | 11.9±0.3 | 213 | >120 | >120 | 51.5 |
| Q143del | 19.8±0.7 | 124 | 14.7±0.5 | 86 | 38 | 3 | 43.5 |

Figure 1. Thermal stability of recombinant wild-type and Q143del mutant P5′N-1. A. Plot of the residual activities at 5 minutes versus temperatures. Wild type (open symbol) and Q143del mutant enzyme (filled symbol) were subjected to heat inactivation in a given range of temperatures. Residual activity is expressed as a percentage of initial activity. B. Far-UV circular dichroism spectra of wild-type (dotted line, 25°C and 37°C) and Q143del mutant enzyme (solid line, 25°C; dashed line, 37°C). Observed ellipticities were converted to mean residue ellipticity (\( \theta \) in deg cm\(^2\) dmol\(^{-1}\)).
It is worth noting that the P5’N deficiency in propositus 2 was found in association with HbD Punjab and Hb Zurich. The presence of associated hemoglobin variants could have exacerbated the clinical pattern in this patient, as reported for HbE. Moreover, this patient had overt iron overload requiring iron chelation. Molecular analysis of the HFE gene showed that she was also heterozygous for the H63D mutation; this mutation, which does not affect iron homeostasis in normal conditions, may result in iron overload in co-operation with other genetic or non-genetic factors.12,20

In conclusion, as already observed, the severity of the phenotype is not directly related to the enzyme activity detected in RBC and this in turn is not a simple and direct consequence of the genotype. 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. Caution should be exercised in drawing prognostic conclusions only from genotypic information.

AZ recruited the patients and contributed to the data interpretation. DCC provided clinical data. EF and PB analyzed hematological data and performed the molecular studies. PA carried out the site-directed mutagenesis analysis. LRC carried out the biochemical experiments. GV and AG contributed to the study design, data interpretation and wrote the manuscript. All authors critically revised the manuscript and approved the final version to be published. None of the authors has any potential conflict of interest. Supported by grants from the University of Pavia Fondi per Ricerca di Ateneo (FAR) and from the Ministero dell’Istruzione, dell’Università e della Ricerca Fondo per gli Investimenti della Ricerca di Base (FIRIB). We thank the “Centro Grandi Strumenti”, Università di Pavia, for free access to the spectropolarimeter. Manuscript arrived March 13, 2006. Accepted July 11, 2006.

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