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Novel homozygous nonsense mutation in the P5′N-1 coding gene as an alternative cause for hereditary anemia with basophilic stippling

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Abstract
Hereditary pyrimidine 5-nucleotidase (P5′N-1) deficiency is a very rare disorder. Here, we describe a new mutation in a Turkish family. Although functional tests have not been performed, our findings confirm that the homozygous mutational state leads to clinical manifest P5′N-1 deficiency, while heterozygosity does not lead to hemolysis or anemia.

KEYWORDS
basophilic stippling, hemolysis, hereditary anemia, pyrimidine 5-nucleotidase (P5′N-1) deficiency

1 | INTRODUCTION

Hereditary pyrimidine 5-nucleotidase type 1 (P5′N-1, cNIII, Cytosolic 5′-nucleotidase 3A) deficiency is a rare autosomal recessive disorder with regard to hemolytic anemia overall, but a common cause of inherited Coombs negative hemolytic anemia. Among chronic non-spherocytic congenital hemolytic anemias, it is the third common disorder besides glucose-6-phosphate dehydrogenase and pyruvate kinase deficiency.[1] It has been documented in over 60 cases since it was first described in the 1970s,[1,2] with a presumably large number of undetected patients.[2] Regarding the known mutations in the encoding *NT5C3A* gene, only patients with homozygous or compound heterozygous mutations develop a phenotype of P5′N-1 deficiency.[2]

Two isoforms of P5′N exist, where the isoform 1 seems to be active mainly toward pyrimidine 5 ribonucleotides, whereas the activity of isoform 2 is not strictly pyrimidine specific and shows more activity toward

deoxyribonucleotides.[3,4] The main function of P5′N-1 is found in the degradation of pyrimidine nucleotides to pyrimidine nucleosides of ribosomal RNA in erythrocytes during the maturation process[5] (Figure 1). More precisely, P5′N-1 catalyzes the dephosphorylation from the RNA nucleotides cytosine monophosphate (CMP)/uridine monophosphate (UMP) to the corresponding nucleosides cytosine and uridine. This process prevents erythrocytes from nucleotide excess[6] (Figure 1), because in contrast to nucleotides, nucleosides can diffuse out of cells and do not accumulate in the cells. P5′N-1 function is entirely dependent on magnesium while lead inhibits its function, and lead intoxication can imitate the clinical manifestation of inherited P5′N-1 deficiency.[6] Hence, it is crucial in the diagnostic workup of suspected inherited P5′N-1 deficiency to exclude lead intoxication as an important differential diagnosis. The main clinical characteristics include (in manner of decreasing frequency) hemolytic anemia, splenomegaly due to extravascular hemolysis, jaundice, and gallstones.[7] Exacerbation of the disease can be caused by infections, and blood transfusions are necessary in distinct cases. Iron overload is only seen in patients who require multiple transfusions.[1] Patients with P5′N-1 deficiency often undergo surgical procedures, with splenectomy being commonly performed in this cohort (about 50%) as well as cholecystectomy, which is less common (~30%).[1]

The hematological findings of inherited P5′N-1 deficiency include signs of hemolysis with raised reticulocyte count, low haptoglobin, and raised levels of bilirubin and lactate dehydrogenase. Cytological workup is elementary and indicative of the diagnosis of P5′N-1 deficiency due to the characteristic red cell basophilic stippling, which is the cardinal cytologic finding and which can be detected in the blood smear due to intracellular accumulation of undegraded RNA.[8] Although this finding is common in P5′N-1, it is not specific and can occur in other diseases occasionally (e.g., beta-thalassemia, some hemoglobin variants, lead intoxication [see above]), so a distinct workup is necessary to define the correct diagnosis.

In this work, we present a case of an adult patient with characteristic basophil stippling in the blood smear leading to the diagnosis of previously not diagnosed P5′N-1 deficiency.

2 | METHODS

2.1 | Case Presentation

A 26-year-old male patient was admitted to our Emergency Department with the diagnosis of an unexplained anemia. He presented with abdominal pain for 48 h and itching of the whole body. Clinical examination revealed jaundice and tenderness to palpation of the upper right abdominal region. Laboratory findings revealed raised serum bilirubin levels (both complete and not-conjugated) with anemia (hemoglobin ranging about 11 g/dl). Abdominal ultrasound showed enlargement of the spleen and intrahepatic cholestasis. The patient underwent an endoscopic retrograde cholangiopancreatography (ERCP) with extraction of two stones and stent implantation. The clinical situation improved, the patient was discharged in good condition and transferred to the outpatient unit for further diagnostic workup.
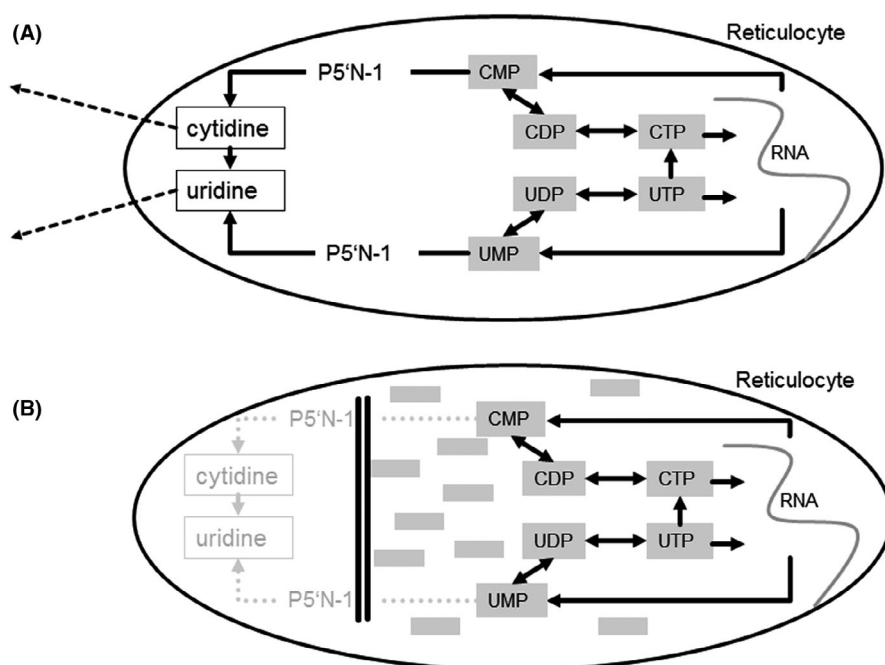

FIGURE 1 (A): Simplified scheme of the pyrimidine catabolism. Uridine monophosphate (UMP) and Cytidine-monophosphate (CMP) are dephosphorylated to uridine and cytidine by P5′N-1. Uridine and cytidine can leave cells via diffusion. (B): Situation with P5′N-1 deficiency (or enzyme block), gray bars indicate accumulation of nucleotides (adapted from Rees et al, Br J Hematol, 2003)

2.2 | Clinical and laboratory findings

Several examinations had been performed to obtain an accurate diagnosis of the—so far unexplained—hemolytic anemia as listed: the patient's history revealed that the anemia had been known since childhood. Prior diagnostics had not elucidated the cause of it, although previous cytological findings include anisocytosis of the erythrocytes, Howell-Jolly bodies, and basophilic stippling. Further laboratory findings with low haptoglobin, raised bilirubin serum levels (s. above), and raised levels of the serum lactate dehydrogenase were in concordance with a hemolytic anemia. MCV and MCH levels showed a macrocytic, hyperchromatic anemia. Furthermore, normal levels for folic acid and cobalamin had been detected with reticulocytosis and high RPI (Reticulocyte production index, RPI >5), which indicated hyperplastic erythropoiesis and no vitamin deficiency.

Detailed information of the blood count and other laboratory findings is given in Table 1. A previously performed direct Coombs test was negative.

2.3 | Hematological diagnostics

The peripheral blood smear revealed a basophilic stippling as characteristic sign of a deficient function of P5′N-1 (Figure 2). Furthermore, no evidence of sickle cell anemia or spherocytosis was present. Bone marrow aspiration revealed erythroid hyperplasia, which was in concordance with the high RPI (see above), but no other abnormal findings. Previous investigations had not revealed any indication of lead intoxication.

Because of the cytological findings, blood samples of the patient were further investigated with regard to inherited causes of hemolytic anemia, especially P5′N-1 deficiency. Enzyme activity of the glucose-6-phosphate dehydrogenase and 6-phosphogluconatdehydrogenase showed normal results, whereas activity of the ribose-phosphate pyrophosphokinase (RPK) was reduced (60%). Reduction of RPK activity is associated with P5′N-1 deficiency,[9,10] so we went forward to genetic evaluation of this case.

2.4 | Genetics

Genomic DNA was isolated from the patient's and relatives′ peripheral blood samples. P5′N gene coding regions and adjacent intronic regions were amplified by polymerase chain reaction, using the primers previously described.[11] Amplified products were subjected to bidirectional sequencing by Sanger's dideoxy chain termination reaction, using the Big-Dye Terminator V.1.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI 3130 Genetic Analyzer (Applied Biosystems).

3 | RESULTS

The patient showed a previously undescribed homozygous insertion of a single A in exon 5 (NM_016489.12:

TABLE 1 Relevant hematologic and other laboratory findings

Laboratory parameters	Findings	Normal range
Hb (g/dl)	11	14–18
HCT (%)	0.33	0.4–0.54
MCV (fl)	113	80–94
MCH (pg)	38	27–32
MCHC (g/dl)	33.5	32–36
RDW (%)	18	11–14%
Reticulocytes (1/1000 erythrocytes)	159	33–110
RPI (reticulocyte production index)	5.69	
Bilirubin total/direct (mg/dl)	7.8	0.2–1
Haptoglobin (g/l)	<0.05	0.3–2.0
Cobalamin (pmol/l)	362	145–637
Folic acid (nmol/l)	>45.4	10.4–42.4
Enzyme activity (μmol substrate turnover/g hemoglobin/min)		
Glucose-6-phosphat dehydrogenase	12.7	11 ± 1.6
6-Phosphoglucogenase	12.3	9.5 ± 1.5
Ribose-phosphate-pyrophosphokinase	58.9	85.8 ± 12.3

c.240dup). This frameshift mutation causes a premature stop signal of translation at codon 82 (p.(Cys81Metfs*2)), with predicted formation of a truncated protein lacking more than 70% of the COOH-terminal amino acid sequence. This variant was neither found in the "1000 genomes" nor the "ExAc" database. However, at the same nucleotide position, the SNP rs745848903 had been identified, consisting of a deletion of the same nucleotide. In fact, it has a frequency of 0.00001 and its clinical relevance was unknown.

Segregation analysis of the family members showed that both parents and one sister were heterozygous for the same mutation, whereas the brother had two wild-type alleles.
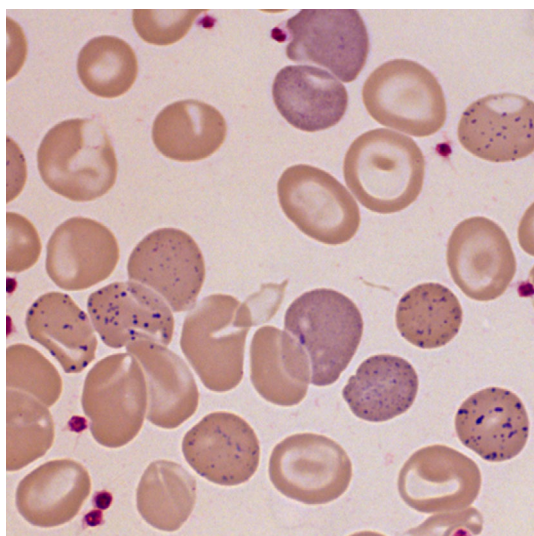

FIGURE 2 Peripheral blood smear, Pappenheim staining. Typical basophil stippling of erythrocytes detected in the described case of P5′N-1 deficiency

4 | DISCUSSION

As stated above, P5′N-1 deficiency is a rare disorder of hemolytic anemia. In the presented case, the cytological diagnostic with the characteristic finding of basophilic stippling enabled us to elucidate the nature of the anemia. During childhood, the patient had been investigated extensively with regard to his hemolytic anemia. A direct Coombs test had been performed previously and had been negative, so all findings were in accordance with P5′N-1 deficiency.

The main differential diagnoses of inherited P5′N-1 deficiency include lead poisoning. As mentioned above, lead can inhibit P5′N-1 function, and many individuals have been described with lead intoxication and notably reduced P5′N-1 function.[12] So, determination of serum lead levels is crucial when basophil stippling is detected cytologically. In our case, previous investigations had excluded lead intoxication. Furthermore, β-thalassemia can also impair P5′N-1 function,[13,14] but in thalassemia, a microcytic, hypochromic anemia is one of the cardinal signs, our patient did not show this kind of anemia. Furthermore, hemoglobin electrophoresis revealed no evidence of thalassemia.

Further molecular-genetic investigations revealed the homozygous c.240-241dup (Figure 3) variant, which causes a truncated protein due to a premature stop signal. A heterozygous mutational state was found in the parents and one sister (Figures 3 and 4). Neither the parents nor the sister showed clinical signs of hemolysis or anemia. Though functional tests of each family member had not been performed, this finding indicates that only the homozygous mutational state leads to clinical apparent P5′N-1 deficiency. According to HGMD database, 26 different mutations had been described in P5′N-1 so far (http://
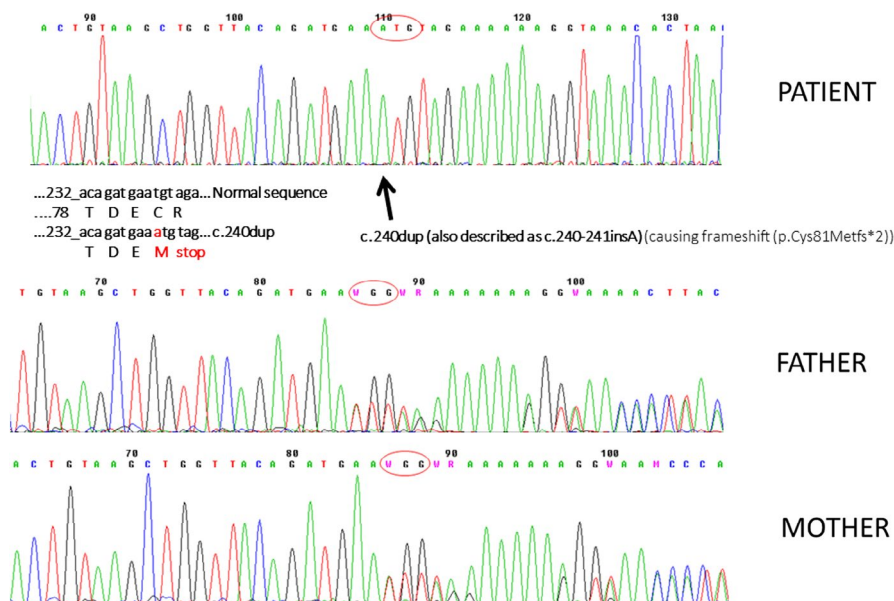

c.240dup (also described as c.240-241insA) (causing frameshift (p.Cys81Metfs*2))

...232_aca gat gaa tgt aga... Normal sequence
....78 T D E C R
...232_aca gat gaa atg tag... c.240dup
T D E M stop

FIGURE 3 DNA sequencing results. Homozygous c.240dup (patient) leading to impaired enzyme function. Heterozygous state detected in parents and in the sister, brother with two wild-type alleles (not shown)
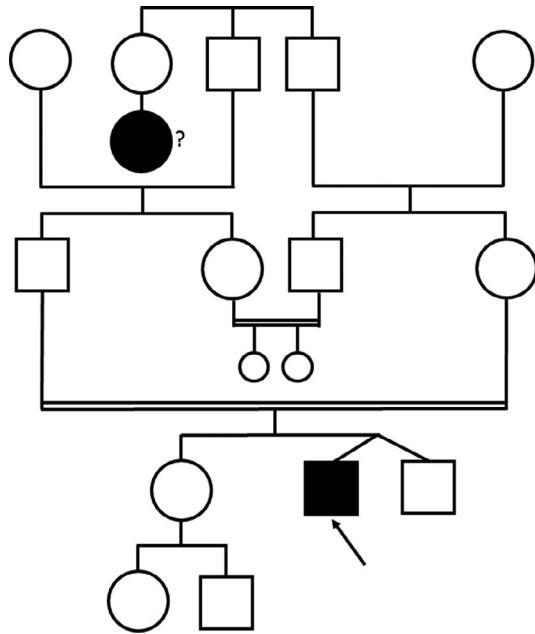
FIGURE 4 Pedigree. There is a consanguinity of parents. The filled symbols (rectangles = male; circle = female) represent the symptomatic family members. The arrow points at our patient. The female relative in the second row was reported to have the same symptoms as our patient, but has not been tested

www.hgmd.cf.ac.uk/ac/gene.php?gene=NT5C3A). The variant described here has not been detected previously. The variant we identified was submitted to ClinVar-NCBI.

There does no specific therapy exist for P5'N-1 deficiency.[6] Supportive measures are the basis of treatment, and need of transfusion is rare. Nevertheless, infections can lead to exacerbation of this disorder and make transfusions necessary.[1] If multiple transfusions are required, monitoring of iron state is recommended and screening for hemochromatosis may be reasonable. Splenectomy had been reported to hardly improve clinical condition in general, with only a few publications reporting transfusion-dependent patients to have become transfusion free after surgery.[15–17]

To summarize, a new variant in the P5'N-1 gene, which very likely leads to an impaired protein function, was found in a patient with hereditary hemolytic anemia. Of note, distinct cytological investigation of the peripheral blood film led us to diagnosis due to characteristic basophilic stippling as cardinal symptom of this disorder. This underlines the importance of thorough peripheral blood smear analysis in anemia of unknown cause.

CONFLICT OF INTEREST
None.

AUTHOR CONTRIBUTIONS
Martin Kirschner analyzed interpreted the data and wrote the manuscript. Inga Rebecca Heinen and Steffen Koschmieder interpreted the data and revised the manuscript. Licinio Manco and Celeste Bento performed genetical analysis and interpreted the data. Thomas Eggermann, Ingo Kurth, Edgar Jost, and Tim H. Brümmendorf interpreted the data and revised the manuscript. Roland Fuchs conceived and planned the report, was involved in the clinical course of the patient, performed cytological workup, and wrote the manuscript.

ETHICAL APPROVAL
None.

CONSENT
Written informed consent was obtained from the patient to publish this report in accordance with the journal's patient consent policy.

DATA AVAILABILITY STATEMENT
For data request, please contact the corresponding author.

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