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Uridine treatment restores the CDA II-like hematological phenotype in a patient with homozygous mutation in the *CAD* gene

Short title: Uridine treatment in a CAD-deficient patient

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A 3-year-old Cambodian male child from consanguineous parents (Figure 1a) presented to neurology with non-verbal autism and intractable seizures. The seizures presented as head nods, drop attacks, or focal dyscognitive events, and they occurred multiple times each day. The electroencephalogram demonstrated aggressive multifocal discharges and a background epileptic encephalopathy.

Investigations using magnetic resonance imaging, lumbar puncture, and standard metabolic testing did not reveal any abnormalities. A partial response to levetiracetam which was not maintained on medication weaning was reported. Seizures had worsened with aging, and multiple anti-epileptics had been used without success. He had undergone recurrent hospital admissions for status epilepticus, decreased mobility with wheelchair use, and autistic regression. Red cell macrocytosis and an abnormal peripheral blood film were reported.

At 6.5 years of age, autism, developmental delay and generalized epilepsy, with partial response to levetiracetam were present. He also exhibited mild to borderline anemia (hemoglobin [Hb], 9.8-10.5 g/dL), relative reticulocytopenia (reticulocyte absolute count, $39.0 \times 10^3/\mu\text{L}$), markedly increased red blood cell distribution width (19.0 %), and signs of hemolysis. He had not received any transfusions. Ferritin and transferrin saturation were within normal limits. Fetal Hb and HbA₂ were increased (Table 1). The peripheral blood smear showed marked anisopoikilocytosis with macrocytes, occasional microcytes and spherocytes, teardrop cells, fragmented red cells, and rare nucleated erythrocytes. The bone marrow aspirate revealed moderate erythroid hyperplasia with

dyserythropoiesis, 5% to 10% binucleated late erythroblasts, rare tri-nucleate forms, and prominent cytoplasmic bridging (Figure 1b). No abnormalities of the fingers, toes, or nails were seen.

The absence of mutations in the β -globin and α -globin genes excluded the presence of a thalassemic syndrome. Acquired causes of dyserythropoiesis were excluded by normal values for vitamin B12, folate, and immunoglobulin D, and a negative human immunodeficiency virus test. The eosin-5-maleimide dye-binding test excluded hereditary spherocytosis. Glucose-6-phosphate dehydrogenase molecular screening was negative. A tentative diagnosis of congenital dyserythropoietic anemia type II (CDA II) was made. This disease is caused by biallelic mutations in the *SEC23B* gene, which encodes a protein of the COPII complex involved in intracellular vesicle trafficking. The most specific finding for CDA II is >10% mature binucleated erythroblasts in bone marrow¹. Most patients with CDA II show hypoglycosylation of band 3 glycoprotein, another specific diagnostic hallmark of the disease². First-line genetic testing was performed with mutational screening of *SEC23B* at 7 years of age, but no known pathogenic variants were identified. At age 11, a second-line genetic testing was performed using a 71-gene custom panel for hereditary anemias³. The genomic DNA preparation, the genetic testing by targeted next-generation (t-NGS) sequencing for hereditary anemias, and the validation of the variants were performed as previously described^{3,4}.

The genomic analysis of the proband highlighted the presence of the ultra-rare missense homozygous variant c.5366G>A, p.Arg1789Gln (rs1341652994, minor allele frequency A = 0.00000 [1/251066, GnomAD_exome]) in the *CAD* gene. SNP-array analysis excluded genomic imbalance associated with autism but showed a loss-of-heterozygosity region that encompassed the *CAD* gene (data not shown).

These data were consistent with a recessive pattern of the inheritance. The same variant was present in both parents in the heterozygous state, with normal hematological phenotypes (Figure 1a).

CDA II-like anemia has also been observed for patients affected by early infantile epileptic encephalopathy-50, a severe neurodegenerative disease that is due to biallelic mutations in the *CAD* gene. This is a very rare condition; since the first family report in 2016, only 4 additional families have been reported so far⁵⁻⁶. Interestingly, all patients affected by this condition also show mild anemia with marked anisopoikilocytosis. *CAD* encodes a multifunctional enzyme complex that comprises glutamine aminotransferase, carbamoyl phosphate synthase, aspartate transcarbamylase, and dihydroorotase, which initiates mammalian *de-novo* pyrimidine biosynthesis by the production of uridine monophosphate. Alternatively, uridine monophosphate can be recycled to uridine through a salvage pathway. Treatment with oral uridine has been shown to be effective for the hematological and neurological phenotypes of CAD-deficient patients⁶. In the first report describing the *CAD* mutation in early infantile epileptic encephalopathy-50, slightly abnormal migrations was for the erythrocyte RhAG protein and band 3 glycoprotein were reported on polyacrylamide gel

electrophoresis, which suggested abnormal glycosylation⁵. Consistent with this finding, we performed western blotting analysis on red blood cell membrane samples isolated from the proband and parents. Western blotting was performed using either rabbit anti-band-3 glycoprotein antibody (H-105, sc-20657; dilution, 1:200; Santa-Cruz Biotechnology, Dallas, U.S.A.) (Figure 1c) or mouse anti-band-3 glycoprotein antibody (dilution, 1:5000; [BIII-136] GTX11012, GeneTex) (data not shown), and rabbit anti-GAPDH antibody (14C10; dilution, 1:1000; Cell Signaling), as previously described⁷.

The immunoblot analysis of isolated erythrocyte membrane proteins from the patient (II.2) and father (I.1) revealed a slight reduction of protein values of band 3 compared to the mother (I.2) and a healthy control, although not statistically significant. However, there was no indication of narrower band size or different migration of band 3 in this patient, compared to a CDA II *SEC23B*-related patient (Figure 1c).

At age 11, treatment with oral uridine supplementation was initiated. Oral uridine (Europepta) was administered as four equal daily doses for a total of 100 mg/kg/day, as previously described⁶. Following 6 months of treatment, the frequency and severity of the seizures were markedly reduced, and the electroencephalogram was no longer encephalopathic although reduced multifocal discharges were evident. Importantly, the patient was weaned off all antiepileptic drugs, became verbal and his social behavior improved. He has learned language picture cards and recognizes letters. He no longer uses a wheelchair. Improvement of the hematological features was also noted (Table 1), which include increased Hb values, normalization of serum haptoglobin, reduction of

HbA₂ and fetal Hb, and of red blood cell distribution width, which returned to within the normal range. Follow-up of the patient after 22 months of uridine treatment demonstrated normal hematological and biochemical parameters, and normal iron balance (Table 1). The blood smear showed normocytic red cells with no significant anisopoikilocytosis (Figure 1B). A bone marrow aspirate showed absence of erythroid hyperplasia, binucleated erythroblasts, or cytoplasmic bridging (Figure 1b).

DISCUSSION

This brief report describes a case of a patient with CDA II-like anemia caused by a rare homozygous variant of the *CAD* gene. Consistent with the other described cases, the patient considered here showed non-verbal autism, intractable seizures, developmental delay, and generalized epilepsy. He also presented mild to borderline macrocytic anemia, relative reticulocytopenia, markedly increased red blood cell distribution width, and signs of hemolysis. The pathogenic variant was located in the same position as a nonsense mutation, p.Arg1789*, which is causative for early infantile epileptic encephalopathy-50⁶. *CAD* was firstly reported to be a candidate gene for congenital disorders of glycosylation affecting the erythrocyte RhAG protein and band 3 glycoprotein⁵. However, in this patient, the immunoblot analysis of isolated erythrocyte membrane proteins did not reveal narrower band size or different migration of band 3 compared to a CDA II *SEC23B*-related patient, but only reduced protein values of band 3. This result was not surprising, as reduced expression of band 3 has also been frequently reported for patients with CDA II⁸.

The identification of the causative genotype, in this case, allowed not only correct diagnosis

but also correct management, with treatment using oral uridine. This case report supports previous data on the beneficial effects of this therapeutic approach in CAD-deficient patients and demonstrates for the first time the long-term efficacy of uridine supplementation for the treatment of patients with this condition.

List of abbreviations

CDA II, Congenital dyserythropoietic anemia type II

Hb, hemoglobin

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Authors' contributions

RR, IA and AI designed and conducted the study, and prepared the manuscript; RM and GDR performed the protein expression analysis; RM contributed to the preparation of the manuscript; FM performed NGS analysis; BER performed the Sanger sequencing; KR and MF take care for the patient and provided critical review of the manuscript. All authors read and approved the final manuscript.

Declarations

The authors declare that they have no competing interests.

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Figure legend

Figure 1. Characterization of a novel uridine-responsive CDA-II-like patient with CAD deficiency. (a) Family pedigree of the patient. Squares, males; circles, females; solid symbols, affected persons; black arrow, the proband. According to the autosomal recessive inheritance pattern, consanguineous parents of the proband (II.2) are heterozygous for the variant c.5366G>A, p.Arg1789Gln in the *CAD* gene. (b) Peripheral blood and bone marrow smears before uridine treatment (left) and after 22 months of uridine treatment (right). (c) Top: representative immunoblot of band 3 glycoprotein in the membrane protein lysate from red blood cells of a healthy control (HC), a CDA II SEC23B-related patient, the proband (II.2), and the parents of the proband (I.1, I.2). Bottom: Densitometric analysis of the blot (n = 4 replicates).

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