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LETTER TO THE EDITOR

Overhydrated hereditary stomatocytosis: A rare cause of familiar persistent macrocytosis due to *SLC4A1* variants

To the Editor:

Defects of red blood cell (RBC) membrane include a heterogeneous group of hemolytic anemias caused by impaired membrane structural organization[1] or altered ion membrane transport, resulting in unbalanced RBC hydration.[2] The most common congenital hemolytic anemia is hereditary spherocytosis (HS), due to defects

TABLE 1 Clinical, hematologic, and biochemical data of the propositus and her mother at the time of the study

	Propositus	Mother	Reference values
Age (years)	17	45	
Neonatal jaundice	Yes	Yes	
Splenomegaly	No	No	
Transfusion (number of units)	0	0	
Hemoglobin (g/dL)	12.1	11.9	12.1–16.7
VGM (fL)	104.7	106.7	78–99
Reticulocytes (10^9/L)	106	82	24–84
Spherocytes (%) Stomatocytes (%)	0 12	0 3	
Unconjugated bilirubin (mg/dL)	0.57	0.74	<1.00
Haptoglobin (mg/dL)	0.62	Nd	30–200
Lactate dehydrogenase (IU/L)	239	164	125–220
Direct antiglobulin test	Negative	Nd	
Indirect antiglobulin test	Negative	Nd	
Osmotic fragility tests			
OF in NaCl on fresh blood	Normal	Increased	Normal
OF in NaCl on incubated blood	Normal	Increased	Normal
Pink test (%)	20	21	11–33
Standard glycerol lysis (seconds)	26	34	23–45
Acidified glycerol lysis (seconds)	119	158	>900
Autohemolysis (%)	2.5	2.8	2–5
+ Glucose	1.0	1.7	0.2–2
+ ATP	1.0	0.8	0.2–2
Ema-binding (% of decrease in fluor)	0	6	>11
SDS–PAGE analysis			
Spectrin/band 3	1.16	0.98	0.97–1.25
Ankyrin/band 3	0.21	0.17	0.10–0.19
Band 4/band 3	0.25	0.25	0.20–0.26

Abbreviations: IU, international unit; MCV, mean cell volume; OF, osmotic fragility; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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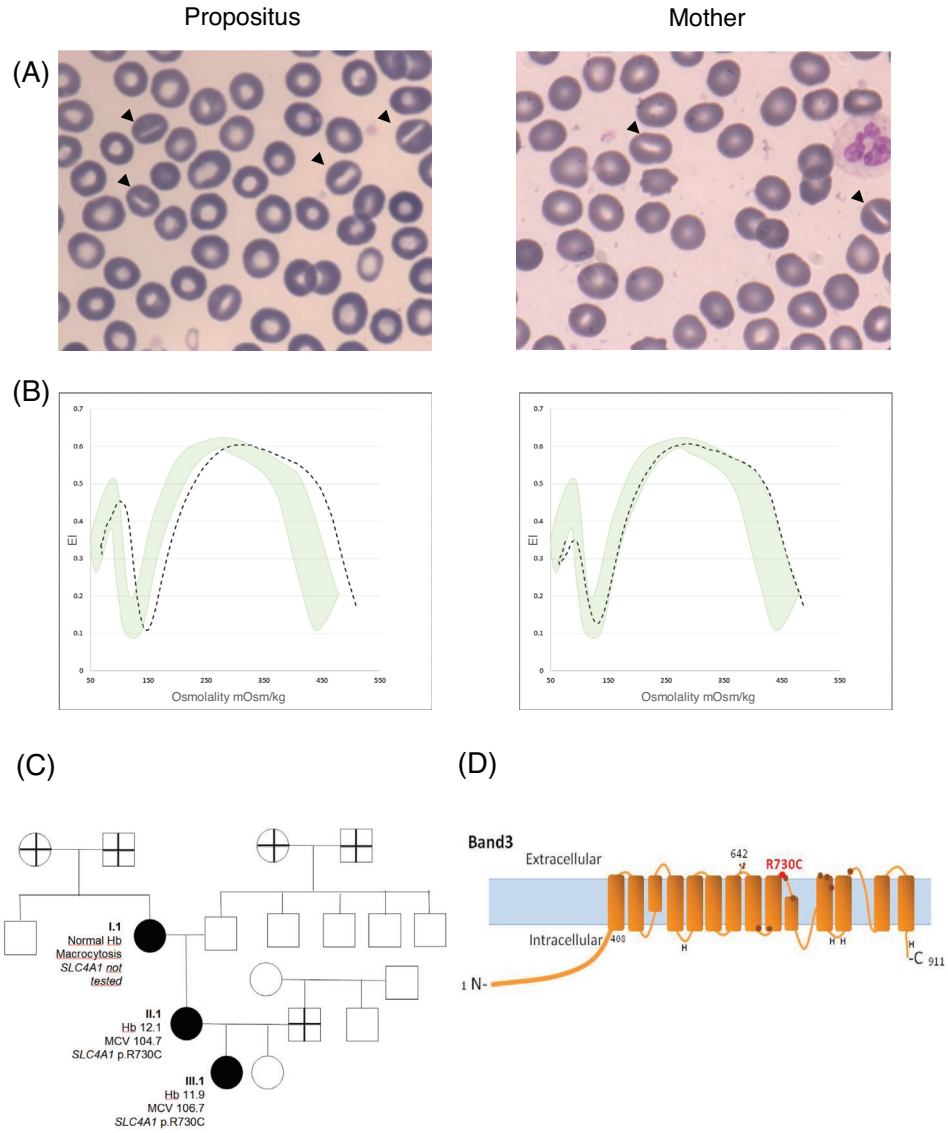

FIGURE 1 (A) Peripheral blood smear of the patient and her mother. May–Grunwald–Giemsa staining, 100×. Stomatocytes are indicated by black arrows. (B) Ektacytometric analysis showing right-shifted curve as typically observed in overhydrated hereditary stomatocytosis (OHSt). Green area represents the area covered by normal controls. (C) Extended family tree. Black circles represent the affected subjects. (D) Schematic representation of the anion exchanger 1 protein and position of p.R730C variant (red). Brown circles represent other residues associated with OHSt.

in genes encoding for key proteins of RBC cytoskeleton, in particular α- and β-spectrin (*SPTA1*, *SPTB*), ankyrin (*ANK1*), protein 4.2 (*EBP42*), and band-3 (*SLC4A1*).[1] Band-3 protein (also known as anion exchanger 1, AE1) has multiple structural functions and is an important chloride/bicarbonate anion exchanger in RBCs.[3] Although band-3 defects are usually associated to HS, mutations in different domains of AE1 protein may result in different phenotypes, as South-East Asian ovalocytosis, or rare forms of hereditary stomatocytosis (HSt).[4] HSt includes a group of hemolytic anemias characterized by increased cation permeability of RBC membrane, resulting in deregulated cellular volume. A particular form, characterized by increased cellular hydration, is known as hydrocytosis or overhydrated stomatocytosis (OHSt), mainly caused by mutations in the *RHAG* gene,[6] even if milder forms have been associated with mutations in *SLC4A1* gene.[4] Being a hemolytic condition, common clinical signs are jaundice, pallor, fatigue, splenomegaly, and gallstones. Due to difficulties in diagnosis, the prevalence of these disorders remains uncertain.[7] However, because of the described high risk of severe thromboembolic complications,[9] the differential diagnosis is particularly important to avoid splenectomy in these patients.

Here, we describe a 17-year-old female who presented to the pediatric hematology outpatient clinic due to persistent unexplained macrocytosis. Born at term, she was hospitalized in neonatal intensive care unit due to severe jaundice, and treated with intravenous (IV) hydration and phototherapy. Macrocytosis was occasionally detected during hospitalization for viral gastroenteritis at the age of 6 years. Nutritional deficiency of folates and vitamin B12 were excluded. Annual routine blood tests did not show any relevant alteration other than macrocytosis (mean cellular volume [MCV] range: 100.5–107.4 fL). Hemoglobin level ranged at the lower limit for

age (10.7–12.6 g/dL), with normal total reticulocyte count, reduced immature reticulocyte fraction (IRF), and relative increase of mature fraction (low fluorescence ratio [LFR]). Further investigations confirmed modest hemolysis, with slightly increased bilirubin, lactate dehydrogenase (LDH) and free hemoglobin, and normal haptoglobin. Coombs test (direct/indirect) resulted negative, as well as screening of abnormal forms of hemoglobin and erythrocyte osmotic fragility test. Erythropoietin, pyruvate kinase (PK), and glucose-6-phosphate-dehydrogenase (G6PD) activity in RBC were normal. No splenomegaly or gallstones were detected at abdomen ultrasound scan. The patient was a second-born girl from non-consanguineous parents. Unexplained macrocytosis (MCV 99.7–106.7 fL) not associated with anemia was previously detected both in her mom and maternal grandmother, but never investigated. In the suspicion of HS, further highly specialized diagnostic investigations were performed in the patient, resulting normal except for acidified glycerol lysis test, which showed increased erythrocyte osmotic fragility (Table 1). Stomatocytes (12%) were detected in peripheral blood (PB) smear (Figure 1A). Ektacytometric osmotic gradient analysis (LoRRca MaxSis, Mechatronics)[10] displayed normal elongation index (EI), with a 15% Omin and 12% Ohyper increase compared to normal controls, resulting in a right-shifted curve suggestive for overhydration (Figure 1B). The eosin 5 maleimide (EMA)-binding test,[11] typically altered in HS, was normal, raising the suspicion of a different form of anemia other than HS. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of RBC membrane did not show any abnormality.[12] A 43-gene next-generation sequencing (NGS)-targeted panel[13] was performed after obtaining patient informed consent, showing the heterozygous pathogenic variant c.2188C>T (p.R730C) of the *SLC4A1* gene (NM_000342). The variant was also identified in the mother by Sanger sequencing, confirming the autosomal dominant inheritance of this defect (Figure 1C).

The p.R730C mutation in the *SLC4A1* gene, reported in literature in another single case,[14] is located within the proposed re-entrant loop 1 (RL1) of the AE1 polypeptide, near a group of previously reported mutations associated with stomatocytosis (p.S731P, p.H734R, p.D705Y, p.E758K).[14–16] RL1 has been proposed as an important determinant of anion selectivity and transport regulation, and mutations in this region may contribute to impair the band-3 function without altering membrane stability or band-3 content.[16] All the patients described carrying mutations in the RL1 domain had an initial diagnosis of HS (or sphero-stomatocytosis due to the presence of stomatocytes).[14,15,17] The clinical features were mild hemolysis, jaundice, splenomegaly, and gallstones in some cases, requiring cholecystectomy and splenectomy. Genotyping allowed to clarify laboratory discrepancies and to finally make the correct diagnosis of OHSt. Although rare, these forms may be underestimated in absence of molecular testing; however, presence of overhydration and abnormal erythrocyte ion content has to be taken into account for patients' management. In this case, based on the confirmed diagnosis of OHSt, disease-specific recommendations were provided to the patient and relatives; in particular, they were informed about the risk of increased thrombotic complications in case of splenectomy.[9]

Concluding, OHSt is a rare form of inherited RBC membrane disorder, which should be suspected in case of persistent otherwise unexplained macrocytosis, especially if present in more members of the same family. Diagnosis of this condition can be difficult and requires third level analyses by a specialized center and genetic testing. The correct diagnosis is important for optimal clinical care and to avoid improper therapy.

AUTHOR CONTRIBUTIONS

Anna Paola Marcello and Francesca Ferrua designed the study and prepared the manuscript. Elisa Fermo and Paola Bianchi performed genetic testing. Cristina Vercellati, Anna Zaninoni, and Anna Paola Marcello performed laboratory investigations on patients' samples. Camilla Visconti, Wilma Barcellini, Alessandro Aiuti, and Francesca Ferrua were responsible for patient's clinical management and follow-up. All the authors provided a critical review of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

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