



PIEZO1 Hypomorphic Variants in Congenital Lymphatic Dysplasia Cause Shape and Hydration Alterations of Red Blood Cells

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PIEZO1 is a cation channel activated by mechanical force. It plays an important physiological role in several biological processes such as cardiovascular, renal, endothelial and hematopoietic systems. Two different diseases are associated with alteration in the DNA sequence of *PIEZO1*: (i) dehydrated hereditary stomatocytosis (DHS1, #194380), an autosomal dominant hemolytic anemia caused by gain-of-function mutations; (ii) lymphatic dysplasia with non-immune fetal hydrops (LMPH3, #616843), an autosomal recessive condition caused by biallelic loss-of-function mutations. We analyzed a 14-year-old boy affected by severe lymphatic dysplasia already present prenatally, with peripheral edema, hydrocele, and chylothoraces. By whole exome sequencing, we identified compound heterozygosity for *PIEZO1*, with one splicing and one deletion mutation, the latter causing the formation of a premature stop codon that leads to mRNA decay. The functional analysis of the erythrocytes of the patient highlighted altered hydration with the intracellular loss of the potassium content and structural abnormalities with anisopoikilocytosis and presence of both spherocytes and stomatocytes. This novel erythrocyte trait, sharing features with both hereditary spherocytosis and overhydrated hereditary stomatocytosis, complements the clinical features associated with loss-of-function mutations of *PIEZO1* in the context of the generalized lymphatic dysplasia of LMPH3 type.

Keywords: *PIEZO1*, lymphedema, red blood cell alterations, overhydration, stomatocytosis, spherocytosis

BACKGROUND

PIEZO1 gene encodes for the mechanoreceptor PIEZO1, a selective cation channel activated by mechanical force (Coste et al., 2010), with several different functions, such as regulation of urinary osmolarity (Martins et al., 2016), control of blood pressure (Wang et al., 2016), or sensor of epithelial cell crowding and stretching (Gudipaty et al., 2017). PIEZO1 is expressed in developing

blood and lymphatic vessels and plays a key role in blood vessel formation (Andolfo et al., 2013; Li et al., 2014; Ranade et al., 2014). Two different diseases are associated with *PIEZO1* mutations: (i) dehydrated hereditary stomatocytosis 1 (DHS1), hemolytic anemia caused by gain-of-function mutations (Zarychanski et al., 2012; Andolfo et al., 2013); (ii) autosomal recessive generalized lymphatic dysplasia with non-immune fetal hydrops (LMPH3) caused by biallelic, loss-of-function mutations (Fotiou et al., 2015; Lukacs et al., 2015). The two diseases are completely different: DHS1 affects red blood cells (RBCs) while LMPH3 is characterized by widespread lymphedema. The only shared phenotype is the presence of perinatal edema (Andolfo et al., 2016; Martin-Almedina et al., 2018).

Several animal models for *PIEZO1* were generated. *Piezo1*-deficient mice die in utero at mid-gestation due to defective vasculogenesis (Cahalan et al., 2015). Thus, another model was developed by a specific deletion in the hematopoietic system (Vav1-*P1cKO* mice). Interestingly, hematological analysis of Vav1-*P1cKO* mice revealed elevated MCV and MCH and reduced MCHC (Cahalan et al., 2015). RBCs exhibited increased osmotic fragility, suggesting that *Piezo1*-deficient erythrocytes were overhydrated. Recently, zebrafish models have also been created. Morpholino-knockdown of *Piezo1* expression in *Danio rerio* was reported to result in severe anemia (Faucherre et al., 2014; Shmukler et al., 2015). However, the phenotype observed in the morpholino-knockdown model was not present in an independent zebrafish model carrying a predicted truncated form of *Piezo1* (Shmukler et al., 2015). The debate on the phenotype observed in the two different models is still open (Shmukler et al., 2016).

Patients with homozygous loss-of-function mutations in human *PIEZO1* show lymphatic dysplasia and an asymptomatic, fully compensated, very mild hemolytic state (Fotiou et al., 2015; Lukacs et al., 2015). Of note, a comprehensible hematological characterization of the anemia carried by patients with *PIEZO1* loss-of-function mutations has not yet been performed. We herein characterized the hematological phenotype of a patient with *PIEZO1* biallelic mutations and lymphatic dysplasia, identifying a new nosological erythrocyte alteration.

CASE PRESENTATION

Patient II.1 (Figure 1A) is a 17-years-old male child affected by non-immune hydrops fetalis and congenital lymphatic dysplasia. During pregnancy, a fetal pleural effusion (32 weeks) was observed. The proband was born at 38 weeks by cesarean section. Birth parameters showed a low Apgar score (5/8) with breathing difficulties treated by continuous positive airway pressure, axial hypotonia, peripheral edema, hydrocele, hypoglycemia, and normal auxologic parameters (weight 3.650 Kg; length 53 cm; and head circumference 36 cm). The hemogram resulted normal for age, and total hyperbilirubinemia was observed (13.2 mg/dL) treated by phototherapy. During childhood, a hydrocelectomy (2-years-old) and a scrotum reduction surgery (14-years-old) were performed. At 14 years, a lower limb lymphoscintigraphy was executed, showing distinctive changes of a severe bilateral

lymphovascular disease. Particularly, the patient highlighted poor asymmetrical uptake of tracer in the groin at 45 min (almost in the right limb) with evidence of rerouting in the scrotum at 2 h. At 15 years, a thoracentesis was performed to reduce the excess of fluid because of respiratory failure due to restrictive lung disease. The cytological analyses highlighted the presence of chylous fluid. After 1 week the chylous edema was re-observed at X-ray. Due to the worsening of respiratory disease at 16 years, magnetic resonance imaging was performed. The analysis showed an impairment of the chylothoraces and reoccurrence of the hydrocele (Figure 1B). Currently, the proband presents a progressive worsening of the respiratory function.

The other family members are healthy expect for the mother of the proband (I.2) that showed an iron deficiency anemia due to imbalanced diet supplies negative for hemoglobinopathies.

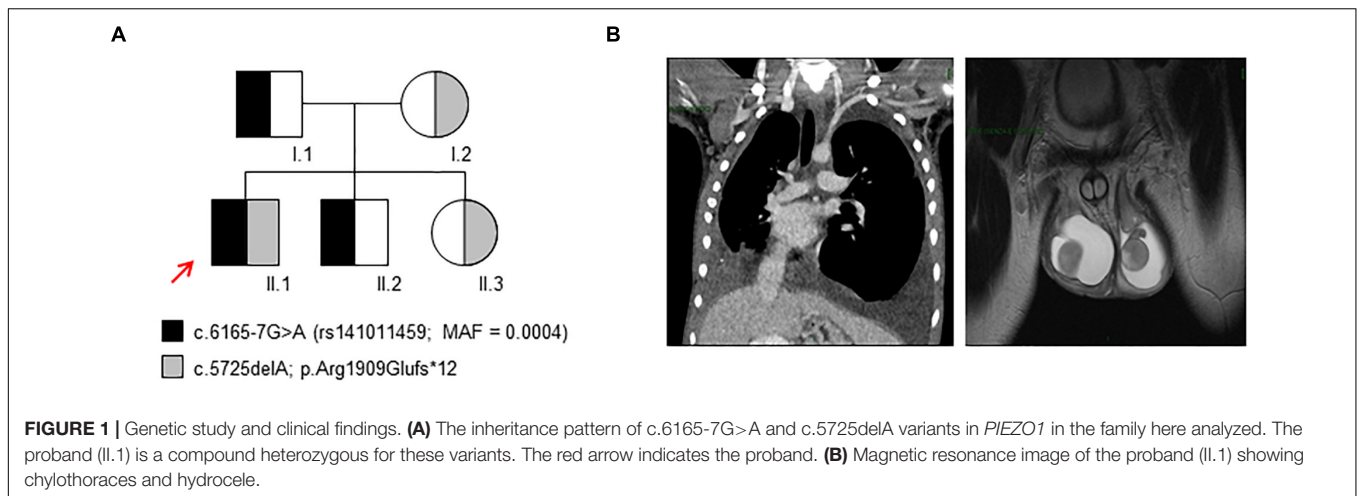
PIEZO1 Mutational Analysis

We performed WES on the proband and the parents, highlighting the presence of two variants within *PIEZO1* gene: the nucleotide substitution c.6165-7G>A in the intron 42–43, annotated in 1000 Genomes database (rs141011459) with a minor allele frequency (MAF) = 0.0004; the novel nucleotide deletion c.5725delA that results in the frameshift variant p.Arg1909Glufs*12 (Figure 1A). According to the recessive pattern of inheritance, the proband showed a compound heterozygous genotype. Indeed, the father, I.1, carried the variant c.6165-7G>A, while the mother, I.2, carried the variant c.5725delA. We also extended the analysis to additional unaffected subjects: the patient's brother, II.2, carried the variant c.6165-7G>A, while the sister, II.3, carried the variant c.5725delA.

To evaluate the possible effect of the frameshift variant on mRNA processing, we sequenced the *PIEZO1* cDNA of the proband. Amplification of the specific exon region, encompassing the mutation, of *PIEZO1* cDNA highlighted the selective expression of the wild-type allele, while the c.5725delA allele was not expressed, demonstrating its decay (Figure 2A). Human Splicing Finder web-tool predicted for the splicing variant c.6165-7G>A the creation of a new “branch point motif,” and two exon splicing enhancer (ESE) motifs for SRp40 protein. High sensitivity analysis of the exon regions encompassing the intronic variant (exons 42–44), using the Agilent 4200 TapeStation system (Supplementary Data Sheet S1), demonstrated that the proband and the father expressed about the 4 and 36%, respectively, of *PIEZO1* cDNA compared to the control (Figure 2B).

Characterization of *PIEZO1* Expression

To further evaluate the role of *PIEZO1* variants, we assessed gene expression in all the family members, as well as in a subset of healthy controls (HCs). A significant decrease of *PIEZO1* expression in the proband compared to those revealed in the HCs was observed, and a minor decrease (about 50%) of mRNA levels in both parents was detected compared to HCs (Figure 2C). Nevertheless, immunoblot analysis on RBCs membranes highlighted a marked decrease of *PIEZO1* protein in the proband compared to the HCs expression with about 30% of expression (Figure 2D). The parents showed also a decrease of *PIEZO1* level with 47 and 65% of *PIEZO1* expression



for mother and father, respectively. Additionally, we evaluated the expression of other RBC membrane proteins, including Band 3 and Stomatins, altered in hereditary spherocytosis (HS) and overhydrated hereditary stomatocytosis (OHS). Proband showed a similar amount of both proteins compared to the HCs (Figure 2E).

Osmotic Fragility Analysis

The ektacytometry analysis was performed for the proband and his parents. As shown in Figure 3A, the proband (II.1) exhibited an ektacytometry curve with right shift compared to the curve obtained from the HCs, indicating overhydration of the erythrocytes. The mother (I.2) showed a right shift of the osmolarity curve similar to those observed in the proband. Conversely, the osmolarity curve of the father I.1 was in the range of the controls with a slight right shift of the curve compared to both the proband II.1 and the subject I.2.

Potassium Content Evaluation

We measured extracellular and intracellular potassium levels in fresh blood samples from all family members, and HCs. The proband (II.1) and his mother (I.2) showed a decrease of potassium content compared to the HC, while the father (I.1) showed intracellular [K⁺] comparable to HC (Figure 3B). The analysis of K⁺ plasmatic levels showed increased levels in the proband and his parents compared to the HC.

Peripheral Blood Smear Examination

The hemogram showed a slight reduction of the Hb content with normal MCV and decreased MCH and MCHC values (Supplementary Table S1). The RDW resulted increased while the reticulocytes count was normal (Supplementary Table S1). Accurate analysis of the peripheral blood (PB) smear of the proband revealed marked anisopoikilocytosis, hypochromia, several spherocytes, some stomatocytes, some mushroom-shaped RBCs, several RBCs fragmentation and debris (Figure 3C).

DISCUSSION

PIEZO1 gene encodes for the mechanoreceptor *PIEZO1*, a selective cation channel activated by mechanical force (Coste et al., 2010; Kim et al., 2012; Ge et al., 2015; Gnanasambandam et al., 2015; Andolfo et al., 2016; Dubin et al., 2017; Hyman et al., 2017; Zhao et al., 2017). In human, the first disease associated with mutations in *PIEZO1* was the DHS1 (Zarychanski et al., 2012; Andolfo et al., 2013). In erythrocytes, *PIEZO1* regulates cell volume homeostasis, and gain-of-function mutations in *DHS1* are causative of alterations of the RBC membrane permeability to monovalent cations Na⁺ and K⁺, with consequent alterations of the intracellular cationic content and cell volume (Albuisson et al., 2013; Bae et al., 2013; Archer et al., 2014; Sandberg et al., 2014; Shmukler et al., 2014; Imashuku et al., 2016). Generally, *DHS1* patients show hemolytic anemia, with high reticulocyte count, the tendency to macrocytosis, and mild jaundice (Zarychanski et al., 2012; Andolfo et al., 2018b). The second condition associated with *PIEZO1* mutations is the lymphatic dysplasia. Two recent reports have described homozygous or compound heterozygous mutations in *PIEZO1* in families with LMPH3 (Fotiou et al., 2015; Lukacs et al., 2015). These cases exhibited full body edema and severe facial swelling. Most patients also presented intestinal lymphangiectasia, growth retardation, seizures, microcephaly, and intellectual disability. Loss-of-function mutations in *PIEZO1* also account for hydrops fetalis, chylothorax, and chronic pleural effusions with persistent lymphedema of legs, torso, and face. The cosegregating homozygous and compound heterozygous *PIEZO1* mutations in these families included non-sense, missense, and splice donor site mutations (Fotiou et al., 2015; Lukacs et al., 2015). Regarding the hematological framework, some of these patients were not anemic and exhibited normal hematological indices, including MCV (Lukacs et al., 2015).

The patient herein described shared some similar characteristics with the other LMPH3 patients until described such as hydrops fetalis, chylothorax, and chronic pleural effusions with persistent lymphedema. On the other hand, our patient showed peculiar characteristics: the hydrocele never

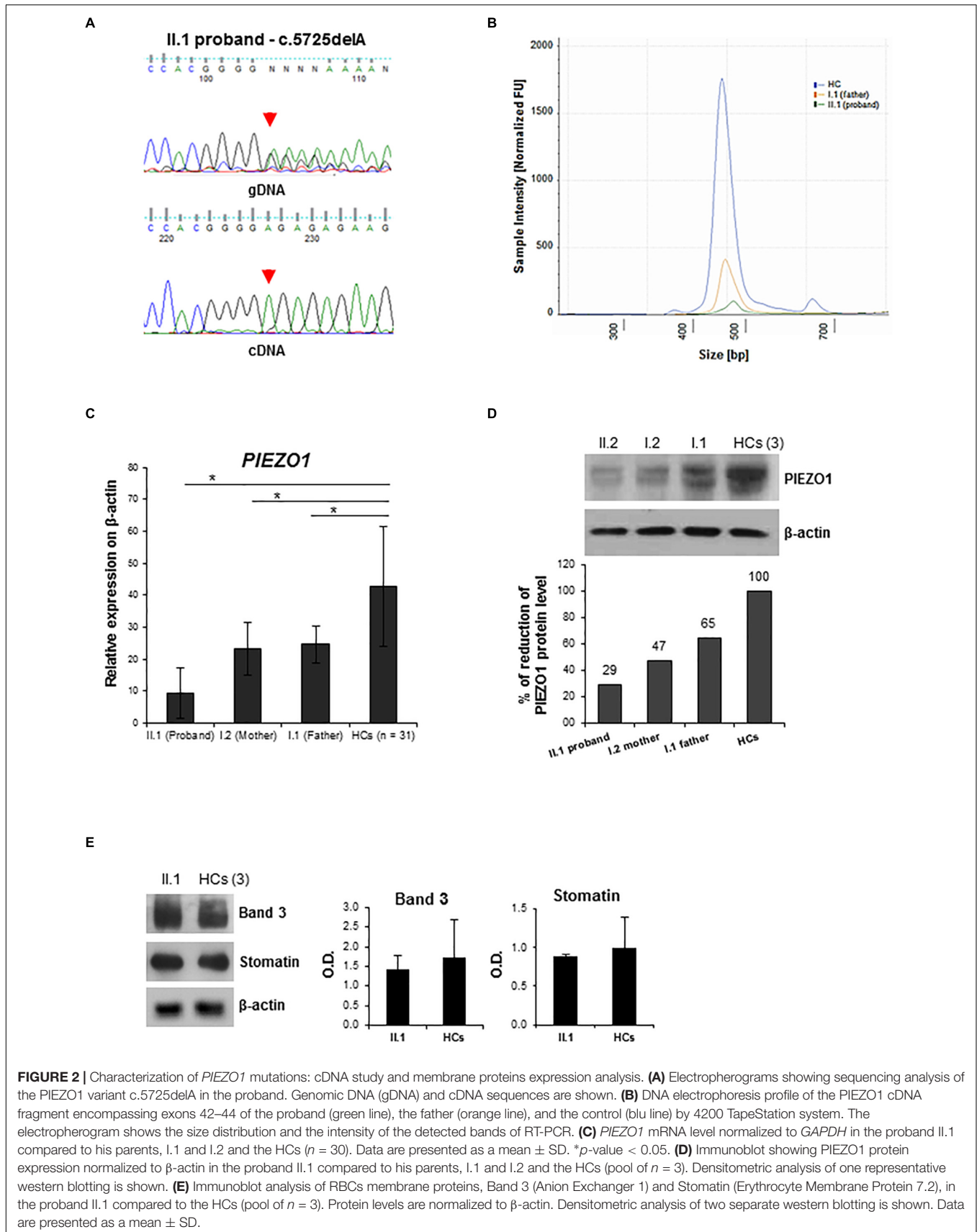
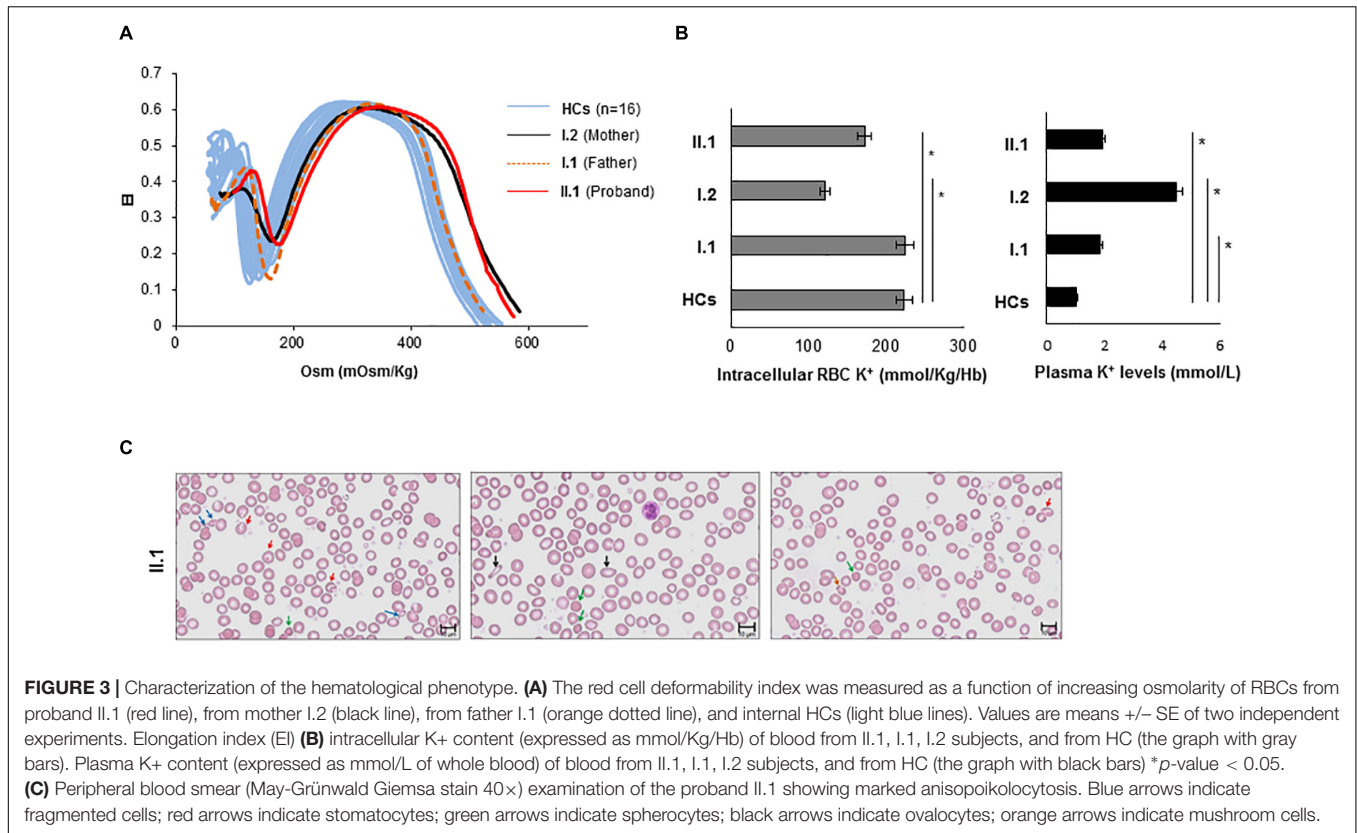


FIGURE 2 | Characterization of *PIEZO1* mutations: cDNA study and membrane proteins expression analysis. **(A)** Electropherograms showing sequencing analysis of the *PIEZO1* variant c.5725delA in the proband. Genomic DNA (gDNA) and cDNA sequences are shown. **(B)** DNA electrophoresis profile of the *PIEZO1* cDNA fragment encompassing exons 42–44 of the proband (green line), the father (orange line), and the control (blue line) by 4200 TapeStation system. The electropherogram shows the size distribution and the intensity of the detected bands of RT-PCR. **(C)** *PIEZO1* mRNA level normalized to *GAPDH* in the proband II.1 compared to his parents, I.1 and I.2 and the HCs ($n = 30$). Data are presented as a mean \pm SD. * p -value < 0.05. **(D)** Immunoblot showing *PIEZO1* protein expression normalized to β -actin in the proband II.1 compared to his parents, I.1 and I.2 and the HCs (pool of $n = 3$). Densitometric analysis of one representative western blotting is shown. **(E)** Immunoblot analysis of RBCs membrane proteins, Band 3 (Anion Exchanger 1) and Stomatatin (Erythrocyte Membrane Protein 7.2), in the proband II.1 compared to the HCs (pool of $n = 3$). Protein levels are normalized to β -actin. Densitometric analysis of two separate western blotting is shown. Data are presented as a mean \pm SD.



observed in the other *PIEZO1* loss-of-function patients, and the absence of facial swelling, lymphangiectasia, and intellectual disability. Of note, the proband is a compound heterozygous for a splicing variant and a coding deletion that causes a premature stop codon. We demonstrated the decay of the allele carrying the deletion variant, and the massive reduction of expression of the allele carrying the splicing variant. The combination of the two variants causes a substantial reduction of both mRNA and protein expression of *PIEZO1* in the proband.

PIEZO1 is a highly polymorphic gene that has a very large tolerance for both missense and loss-of-function variants and has a lot of variations. The variable expressivity of both DHS1 and lymphatic dysplasia could be explained with the combination of multiple disease-causing alleles or their combination with polymorphic variants (Lupski, 2012; Lacroix et al., 2018). Indeed, we previously demonstrated that multiple modifier *PIEZO1* variants could account for highly variable clinical expressivity in DHS1, with subsequent difficulties in establishing the appropriate genotype/phenotype correlation (Andolfo et al., 2018a,b). Of note, the patient showed a peculiar phenotype characterized by peripheral edema, hydrocele, and chylothoraces. Furthermore, even if the blood count seems only slightly altered with a mild reduction of the Hb, and decreased MCH and MCHC values, the RDW resulted increased despite the reticulocytes count was normal. According to the increased RDW, the PB smear of the proband revealed anisopoikilocytosis, hypochromia, with the presence of some spherocytes, mushroom-shaped RBCs, stomatocytes, erythrocytes' fragmentation, and debris. Moreover,

the ektacytometry analysis revealed a right shift of the right arm of the osmolarity curve indicating mild overhydration of RBCs, without the decreased DIMax typical of HS. Finally, the ionic flux assay indicated increased plasma [K⁺] and decreased intracellular [K⁺] as in OHS. Thus, our patient seems to present pathological traits of the erythrocyte with some characteristics shared with hereditary spherocytosis as spherocytes at PB smear and normal MCV and several features of overhydrated hereditary stomatocytosis as stomatocytes at PB, decreased MCHC, normal Dimax, right shift of the osmolarity curve, and decreased intracellular potassium. The mother showed a similar, but less pronounced, right shift of the osmolarity curve. This finding could be caused by the iron deficiency anemia that is known to alter the deformability of RBCs (Vayá et al., 2005; Brandão et al., 2009).

Of note, Vav1-P1cKO mice with specific deletion of *Piezo1* in the hematopoietic system showed a slight increase of RDW and reduced MCHC confirming overhydration of RBCs as seen in our patient (Cahalan et al., 2015). Moreover, morpholino-knockdown of *Piezo1* in zebrafish showed the erythroid phenotype of fragile, spherocytic, dysmorphic cells also like our patient (Shmukler et al., 2015).

In conclusion, the proband presents an alteration of the structure and the ionic content of erythrocytes caused by the two hypomorphic variants in *PIEZO1*. We speculate that the substantial decreased expression of *PIEZO1* could be compensated by overactivation of other cation channels/pumps that act by compensating the hematological phenotype. Patients

affected by lymphedema caused by mutations in *PIEZO1* could benefit in future of therapy by Yoda1, a novel small synthetic molecule specific activator of *PIEZO1* (Cahalan et al., 2015; Lacroix et al., 2018), or by gene therapy by selective insertion of the gene in the lymphatic system, or by *in vivo* target gene activation via CRISPR/CAS9 mediated *trans*-epigenetic modulation.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

Ethics Committee of University Federico II, number 197/18.

AUTHOR CONTRIBUTIONS

IA, RR, and AI designed and conducted the study, and prepared the manuscript. GDR performed the western blotting analysis

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and contributed to the preparation of the manuscript. EE and AV performed the preparation of the WES libraries and the NGS analysis. FM and BER performed the molecular analysis and collection of the samples. AG, VC, and GP contributed to take care of the patients. LDF performed the ionic flux data analysis. RR performed the mutational analysis. OZ designed and supervised the NGS analysis and also provided a critical evaluation of the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2019.00258/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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