

PIEZO1-R1864H rare variant accounts for a genetic phenotype-modifier role in dehydrated hereditary stomatocytosis

Dehydrated hereditary stomatocytosis (DHS) is an autosomal dominant hereditary hemolytic anemia characterized by erythrocyte dehydration due to loss of the cation content. Affected subjects exhibit highly variable clinical presentation, ranging from absence of clinical symptoms to lethal perinatal edema. They may present severe iron overload leading to hepatic transplantation, or life-threatening thromboembolic disease after splenectomy, thus making the diagnosis of this condition problematic.¹ DHS results in two different forms: i) DHS1, the most frequent, is caused by mutations in *PIEZO1*, encoding a cation selective channel activated by mechanical force; ii) DHS2 due to an altered *KCNN4* gene, encoding a Ca²⁺-sensitive (Gardos) channel.²⁻⁴ In particular, *PIEZO1* is a large and highly polymorphic gene. Several electrophysiology studies demonstrated that the mutations cause a gain-of-function phenotype with delayed inactivation of the channel.⁵⁻¹⁰

We studied 7 DHS patients from two unrelated families (A-B) showing highly variable clinical expressivity and carrying the same new *PIEZO1* mutation. We demonstrated that the presence of an additional *de novo* *PIEZO1* rare missense variant in one of the 2 probands accounts for a more severe phenotype.

Case 1 from Family A (A-I1) was a 42-year old male from the UK who had suffered from hemolytic anemia for over 20 years. Four of his 5 children also suffered from anemia (Figure 1A). He had gallstones and splenomegaly. On complete blood count (CBC), decreased hemoglobin (Hb) levels, and increased mean cell volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and reticulocyte count were observed (Table 1). He also exhibited high levels of bilirubin and lactate dehydrogenase (LDH), as well as increased ferritin and transferrin saturation, indicating iron overload (negative for hemochromatosis) with normal liver function. Peripheral blood smear highlighted the presence of macrocytosis, polychromasia, schistocytes and few stomatocytes.

Case 2 from Family B (B-II2) was a 6-year old child from Southern Italy (Figure 1B). At birth, he presented jaundice that had been treated with phototherapy. At seven months, he was hospitalized for skin and scleral jaundice. During this hospitalization, severe normocytic anemia with reticulocytosis was observed. At three years, he experienced severe anemia (Hb 8.3 g/dL), with reticulocytosis (181764/mL, 6.12%), high platelet count (662x10⁹/L), signs of hemolysis (high unconjugated bilirubin and low haptoglobin levels), splenomegaly (longitudinal diameter 10.5 cm). Iron balance was normal for his age (transferrin saturation 18%, ferritin 64 ng/mL). He was hospitalized several times after the age of three years, requiring frequent transfusions (2-3 per year) because of hemolytic crisis. Peripheral blood smear highlighted the presence of polychromasia, schistocytes and few stomatocytes.

The father B-I1 was also affected, but he showed a well-compensated anemia with normal Hb (14.6 g/dL), macrocytosis (MCV) (98.2 fL), reticulocytosis (453100/mL, 11.53%), and splenomegaly (longitudinal diameter 16.5 cm). Unlike his son, he did not show symptomatic anemia either in childhood or in adulthood; only a very mild anemia at eight years of age, observed during routine CBC evaluation, had been reported. Thus, patient B-II2 exhibited a more severe phenotype with a neonatal presentation (at birth compared to 20 years for patient A-I1 and 8 years for patient B-I1) and lower Hb levels (8.7±0.3 g/dL) com-

Table 1. Blood count and laboratory data of A-I.1, B-II.2 and probands.

	A-I1	B-I1	B-II2	Normal range
Onset symptoms (years)	20	8	At birth	
Age at diagnosis (years)	42	37	3	
Complete blood count				
RBC (x10 ⁹ /L)	5.1	4.48	2.97	4.2-5.6
Hb (g/dL)	9.5	12.9	8.3	12-17.5
MCV (fL)	99	99.1	73.6	80-97
MCHC (g/dL)	39.7	37.4	38.0	32-38
Ret (%)	3.2	10.5	6.12	0.5-2
Laboratory data				
Total bilirubin (mg/dL)	9.6	–	2.9	0.2-1.1
Serum iron (µg/dL)	234	–	71	45-150
Ferritin (ng/mL)	646	–	62	5-150

RBC: red blood cell count; Hb: hemoglobin; MCV: mean cell volume; MCHC: mean cell hemoglobin concentration; Ret: reticulocytes.

pared to both the previous cases: A-I1, 9.5 g/dL, and his father B-I1, 12.9 g/dL (Table 1).

Ektacytometric analyses were performed for all the subjects reported here. As shown in Figure 1C and D, all affected subjects from Family A and the patient B-I1 exhibited a similar leftward shift of the bell-shaped curve compared to the curves obtained from unaffected subjects from both families and healthy control (Omin: patients 135±7, controls 153±7.6, $P=0.003$; Ohyper: patients 429±12, controls 487±23, $P=0.001$) (Online Supplementary Table S1), indicating dehydration of the red blood cells (RBCs) (Figure 1C and D). Of note, the proband B-II2 showed a more pronounced left shift of the curve compared to both his affected father B-I1 and the other patients of the family A (Omin: overall patients 135±6.5, B-II-2 96.5±0.7, $P=0.0006$; Ohyper: overall patients 429±12, B-II.2 416±4.2) (Online Supplementary Table S1). Thus, patient B-II2 showed a more severe dehydration of RBCs.

The clinical profile aroused the suspicions of DHS, and the direct sequencing of the *PIEZO1* gene was carried out in both families. (See Online Supplementary Appendix for further details). Affected subjects of Family A revealed the heterozygous missense mutation c.7473_7478dupGGAGCT, leading to the in frame duplication of two amino acids, p.E2492_L2493dup, localized in the C-terminal intracellular domain (CTD) that forms the pore of the channel mediating the ion conduction and the cation selectivity.^{11,12} This mutation was absent in the unaffected subjects of the same family. We found the same duplication also in Family B (Figure 1A and B). A similar mutation in *PIEZO1*, p.E2496ELE, had been previously described with a frequent recurrence (8 of 11 patients described), confirming that this region is a hot spot for mutations.⁶ In particular, as previously assumed, the in-frame insertion of six nucleotides (c.7473_7478dupGGAGCT) surrounded a low complexity sequence between positions 2491 and 2499, and slippage mutagenesis leads to the addition or removal of one copy of a short tandem repeat. This mechanism of microdeletion or microinsertion is frequently described in the human genome when there are short tandem repeats similar to the sequence surrounding the duplication.⁶

Interestingly, the proband B-II2 also carried an additional *PIEZO1* variant c.5591G>A, p.R1864H (rs770843415) with very low minor allele frequency (MAF) [$T=0.0011$ (ExAC);

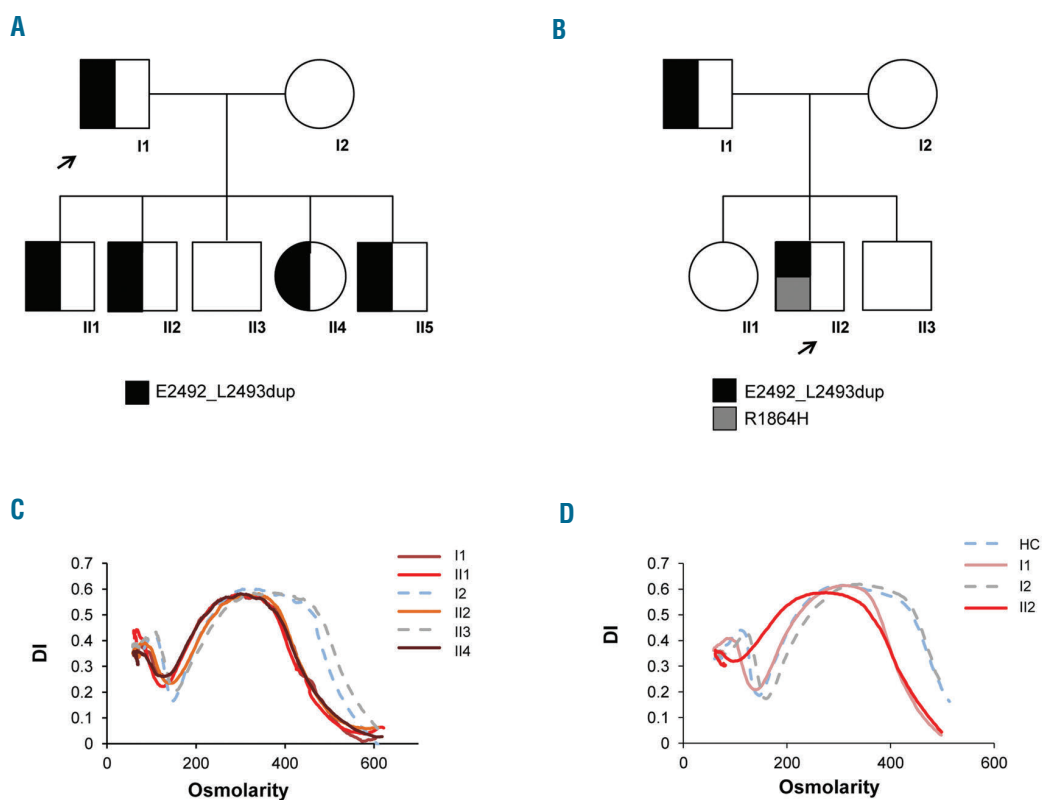


Figure 1. Partial pedigree of the two families analyzed and ektacytometry analyses. (A) Inheritance pattern of *PIEZO1*-E2492_L2493dup mutation in Family A. The proband A-I1 is indicated by an arrow. (B) Inheritance pattern of *PIEZO1*-E2492_L2493dup mutation in Family B. The proband B-II2 is indicated by an arrow. (C) The red cell deformability index (DI) was measured as a function of increasing osmolarity red cells from Family A patients I1, I2, II2, IV2 and from internal healthy controls of the same family (I2 and II3). Values are means \pm Standard Error of Mean (SEM) of two independent experiments. (D) DI was measured as a function of increasing osmolarity red cells from family B patients I1, 2II, from unaffected subject of the same family (I2), and from a healthy control. Values are means \pm SEM of two independent experiments.

T=0.0004 (TOPMED)]. The variant was predicted to be tolerated by PolyPhen2 and SIFT, and possibly pathogenic by M-CAP (score 0.849). Of note, the analysis of inheritance pattern in Family B demonstrated that R1864H is a *de novo* event occurring in the proband B-II2. Interestingly, the residue R1864 localized in the non-pore containing region of *PIEZO1* that confers mechanosensitivity to the channel.^{11,12} Thus, the additional variant R1864H could also impair the mechanosensitivity pathway mediated by *PIEZO1*. By means of a cloning assay, we tested the segregation of both *PIEZO1* mutations in each allele of patient B-II2, demonstrating that both variants, E2492_L2493dup and R1864H, were on the same allele. Of note, the proband B-II2 showed a more severe phenotype compared to his affected father (B-I1) and to the affected subject A-I1 (Table 1). Thus, we speculate that the co-inheritance of both causative and modifier variants in this patient could account for the variable expressivity of his phenotype. We further analyzed other possible genes involved in the dehydration pathways of RBCs, such as *KCNN4* and *ABCB6*, but we found no additional mutations in these genes.

In order to evaluate the role of *PIEZO1*-R1864H variant as phenotype-modifier, we modeled *in vitro* the genotypes of our patients by transient transfection of wild-type (WT) and mutant *PIEZO1* expression plasmids into HEK293 cells. No significant differences among mutants, E2492_L2493dup and R1864H, and WT *PIEZO1* mRNA

were observed 72 hours post transfection. However, the co-inheritance of the two variants E2492_L2493dup + R1864H on the same allele resulted in a slight reduction in mRNA expression (Figure 2A). Nevertheless, immunoblot analysis of *PIEZO1* showed equivalent expression of WT and mutant *PIEZO1* polypeptides (Figure 2B). In addition, immunofluorescence analysis demonstrated that none of the mutants impaired the plasma membrane co-localization with the cell mask plasma membrane marker (*Online Supplementary Figure 1S*). When we analyzed the activity of the channel by measurement of ouabain- and bumetanide-resistant net cation flux, as previously described,¹³ we observed a greater loss of cell K^+ from mutants *PIEZO1*-expressing cells compared to WT *PIEZO1*-expressing cells. Of note, the presence of the two variants E2492_L2493dup + R1864H *in cis* led to an increase of K^+ efflux with respect to each variant in heterozygous state (*PIEZO1*-E2492_L2493dup and *PIEZO1*-R1864H) (Figure 2C). Correspondingly, residual intracellular Rb^+ content was significantly reduced in cells expressing *PIEZO1* mutant genotypes compared to WT *PIEZO1* (Figure 2D). Our functional study revealed the causative effect of both novel mutations here described. Moreover, we provide here for the first time molecular and functional evidence of the phenotype-modifier role of the *de novo* mutation R1864H, co-segregating with the inherited E2492_L2493dup, on the occurrence of severe phenotype observed in the proband B-

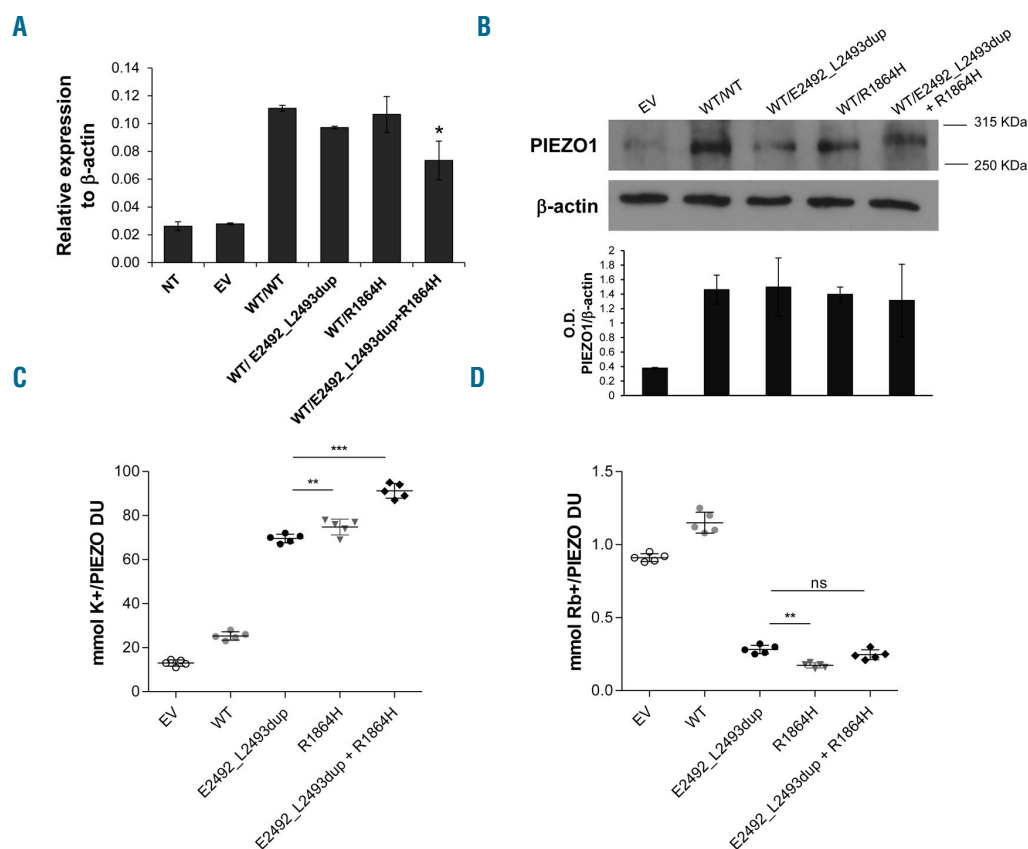


Figure 2. Modifier effect of PIEZO1 R1864H on cation flux. (A) PIEZO1 mRNA levels in HEK-293 cells over-expressing human PIEZO1-WT, PIEZO1-E2492_L2493dup, PIEZO1-R1864H, and PIEZO1 double mutant E2492_L2493dup + R1864H. Values are means \pm Standard Error of Mean (SEM) of three independent experiments. * $P < 0.05$ WT/WT vs. WT/E2492_L2493dup+R1864H; WT/E2492_L2493dup vs. WT/E2492_L2493dup+R1864H; WT/R1864H vs. WT/E2492_L2493dup+R1864H. (B) (Top) Immunoblot showing PIEZO1 protein expression in cells over-expressing human PIEZO1-WT and mutants. β -actin is loading control. (Bottom) Densitometric analysis of three separate Western blotting with similar results. (C) Extracellular K⁺ content from HEK-293 cells over-expressing PIEZO1-WT and mutants, as described in (A). Open circles indicate empty vector (EV); gray circles indicate wild-type (WT); black circles indicate PIEZO1-E2492_L2493dup; gray triangles indicate PIEZO1-R1864H; black rhomboids indicate PIEZO1 double mutant E2492_L2493dup+R1864H ($P < 0.0001$ all mutants by ANOVA test; *** $P < 0.0001$ E2492_L2493dup vs. E2492_L2493dup+R1864H by Bonferroni *post-hoc* test; ** $P < 0.05$ E2492_L2493dup vs. R1864H by Bonferroni *post-hoc* test). Values are depicted for each replicate (n=5). (D) Cell Rb⁺ content of cells over-expressing PIEZO1-WT and mutants, as described in (C). Open circles indicate EV; gray circles indicate WT; black circles indicate PIEZO1-E2492_L2493dup; gray triangles indicate PIEZO1-R1864H; black rhomboids indicate PIEZO1 double mutant E2492_L2493dup+R1864H ($P < 0.0001$ all mutants by ANOVA test; ** $P < 0.05$ E2492_L2493dup vs. R1864H by Bonferroni *post-hoc* test; E2492_L2493dup vs. E2492_L2493dup+R1864H by Bonferroni *post-hoc* test). Values are depicted for each replicate (n=5). ns: not significant.

II2. In particular, the effect mediated by R1864H is mainly evident in the modulation of the hydration status of RBCs. Indeed, we clearly demonstrate that this missense rare variant accounts for an augmented K⁺ efflux when co-inherited with the duplication, leading in turn to more severe clinical presentation of the disease, as observed in patient B-II2.

This finding highlights the importance of studying the effect of multiple modifier *PIEZO1* variants on the genotype-phenotype correlation, since it is by now well-known that many Mendelian disorders could be explained by the combinations of multiple disease-causing alleles, or their combination with polymorphic variants.^{14,15} Indeed, the occurrence of complex genotypes could explain the highly variable clinical expressivity witnessed in DHS patients, with subsequent problems in establishing the appropriate genotype/phenotype correlation. It is worthy of note that the study of *PIEZO1* polymorphic variants could be significant also for other anemias characterized by dehydration, such as sickle cell disease.

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