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# Hereditary elliptocytosis-associated alpha-spectrin mutation p.L155dup as a modifier of sickle cell disease severity

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## Abstract

The broad phenotypic variability among individuals with sickle cell disease (SCD) suggests the presence of modifying factors. We identified two unrelated SCD patients with unusually severe clinical and laboratory phenotype that were found to carry the hereditary elliptocytosis (HE)-associated alpha-spectrin mutation c.460_462dupTTG (p.L155dup), a mutation enriched due to positive selective pressure of malaria, similar to the SCD globin mutations. High index of suspicion for additional hematologic abnormalities may be indicated for challenging patients with SCD. These cases highlight the validity of specialized testing such as ektacytometry and Next-Generation sequencing for patients and family members to assess genotype/phenotype correlations.

### Keywords

sickle cell disease; alpha-spectrin; hereditary elliptocytosis; hereditary pyropoikilocytosis; ektacytometry; Next-Generation sequencing

## INTRODUCTION

Sickle cell disease (SCD) is a phenotypically variable disease characterized by polymerization of the mutant hemoglobin under deoxygenating conditions. Sickle red blood

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The authors declare no competing financial interests.

cells (RBCs), after repeated cycles of deoxygenation/oxygenation, become irreversibly misshapen and increasingly susceptible to hemolysis. Damage to the RBC cytoskeletal proteins as a result of increased levels of reactive oxygen species (ROS) contributes to this phenomenon.[1,2] Coinheritance of RBC cytoskeletal defects could be one of the sources of heterogeneity in SCD.

Hereditary elliptocytosis (HE) and pyropoikilocytosis (HPP) are RBC cytoskeletal disorders with increased prevalence in population of African origin, presumably due to positive selective pressure of malaria for the causative mutations in the genes encoding alpha-spectrin (*SPTA1*), beta-spectrin (*SPTB*), and protein 4.1R (*EPB41*). These mutated proteins result in defective horizontal association of the RBC cytoskeleton, leading to decreased membrane deformability and stability and consequently to increased hemolysis.[3,4]

We describe two unrelated SCD patients with unusually severe hemolysis, suggesting the possibility of an additional modifying factor. Using Next-Generation sequencing of genes associated with RBC membrane disorders we determined that the patients were heterozygous for the hereditary elliptocytosis (HE)-associated *SPTA1* mutation p.L155dup.[5]

## METHODS AND RESULTS

All study subjects were enrolled in our IRB-approved Hereditary Hemolytic Anemia Study and consented to provide blood samples for sequencing and additional testing. Patient 1 was a 2-year-old African American male diagnosed with SCD by newborn screening and confirmed to have genotype SS. He was started on hydroxyurea (HU) at 16 months of age with a personalized starting dose through the Therapeutic Response Evaluation and Adherence Trial[6] (TREAT, ClinicalTrials.gov identifier: NCT02286154), and sustained hemoglobin F (HbF) values of greater than 25% indicating good compliance. Despite robust HbF response, he continued to experience persistent hemolysis characterized by hemoglobin values as low as 6 to 7 g/dL, reticulocytes of 10 to 16.5% (absolute reticulocyte count [ARC] 230–340 $\times 10^3$/μl), and nucleated RBC counts up to 5–12%. Examination of blood smears before and, more clearly, after HU treatment revealed the presence of elliptocytes amidst few sickled cells (Figures 1A and 1B). Ektacytometry (Lorrca, Mechatronics Instruments) was performed before initiation of HU and after the patient reached maximum tolerated dose (MTD). Ektacytometry measures RBC deformability while samples are exposed to a constant shear stress of 30 Pa and an increasing osmotic gradient.[7,8] The patient's ektacytometry curve at baseline (prior to treatment with HU) showed decreased RBC deformability (low Elongation Index [EI]), a typical finding in SCD (Figure 1C). Although RBC deformability improved with HU, reaching a higher EI, the patient demonstrated only a modest improvement in his RBC deformability in comparison to other patients with SCD with good compliance to HU treatment (Figure 1D). Sequence analysis of 12 genes (RBC Membrane Disorders Panel including *ABCG5*, *ABCG8*, *ANK1*, *EPB41*, *EPB42*, *PIEZO1*, *RHAG*, *SLC2A1* [*GLUT1*], *SLC4A1*, *SPTA1*, *SPTB*, and *XK*) revealed that the patient was heterozygous for the *SPTA1* c.460_462dupTTG (p.L155dup) mutation (Table 1 and Figure 1F). Ektacytometry of a blood sample from the patient's mother demonstrated a trapezoidal curve typical of HE[9] (Figure 1E), despite a clinically unremarkable hematologic

history. Targeted sequencing of DNA revealed that she was heterozygous for the *SPTA1* c.460_462dupTTG (p.L155dup) mutation (Table 1 and Figure 1F).

Patient 2 was a female with SCD (genotype SD) evaluated at 5 months of age. Her father was of African American and Puerto Rican heritage and heterozygous for HbS, and her mother was of European American (Greek) origin and heterozygous for hemoglobin D (HbD) Los Angeles (also known as HbD Punjab).[10] A blood smear in infancy prior to any transfusion showed anisocytosis, poikilocytosis, sickle cells, and elliptocytes. The patient had sequencing analysis using the RBC Membrane Disorders Panel due to a more pronounced anemia than expected at her age, splenomegaly, and a blood smear consistent with hereditary pyropoikilocytosis (HPP). She was found to be heterozygous for the *SPTA1* c.460_462dupTTG (p.L155dup) mutation and heterozygous for the low expression *SPTA1* α−LELY variant (c.6531–12C>T and the linked polymorphism c.5572C>G [p.L1858V])[11] (Table 1 and Figure 1F). Targeted sequencing of DNA from the parents revealed that the father was heterozygous for the *SPTA1* c.460_462dupTTG (p.L155dup) mutation and both parents were heterozygous for *SPTA1* α−LELY (Table 1 and Figure 1F). Ektacytometry was not evaluable for this patient since she was receiving frequent transfusions, but was performed on blood samples from the parents and revealed that the father had a profile consistent with HPP (Figure 1G), suggesting that the *SPTA1* HE mutation was in trans to the α-LELY variant in the father and consequently in Patient 2 as well (inherited from her mother). However, we were unable to confirm this with genetic testing since there were no additional variants in the vicinity of the variants of interest that could be used for phase determination by sequencing of the parental samples, and additional family members were not available for genetic testing.

## DISCUSSION

The considerable phenotypic variability of SCD suggests modifying pleiotropic and epistatic effects that may originate from RBC components other than hemoglobin and from surrounding tissues and cells.[12] One source of heterogeneity could be additional RBC disorders including cytoskeletal defects. We describe two SCD patients with unusually severe phenotypes who were heterozygous for the HE-associated *SPTA1* mutation p.L155dup.[5]

Alpha-spectrin mutation p.L155dup is a common but interesting variant first described in several unrelated European and American families with African heritage.[13,14] Located in the region of the spectrin self-association site, it confers an impaired ability to form tetramers, usually without a significant reduction in the amount of spectrin associated with the membrane. Individuals heterozygous for this mutation typically have a compensated mild HE, elliptocytes in peripheral blood smears, and the classic trapezoidal HE ektacytometry curve.[13–16] Interestingly, an epidemiological study of the spectrin mutations related to HE conducted in Benin revealed a dramatically higher incidence of these mutations in multiple African ethnic groups compared to previous studies in the Caucasian population, suggesting that such mutations might have been positively selected due to the high incidence of malaria in these groups.[17] In addition, RBCs from individuals with the p.L155dup mutation showed

a decreased invasion by *Plasmodium falciparum* which correlated with the percentage of spectrin dimers present in the RBC membranes.[18]

Weakening of the spectrin-based cytoskeleton by the p.L155dup mutation would be expected to contribute to the aggravated hemolysis observed in both affected SCD patients. In addition, Patient 2 was heterozygous for the low-expression *SPTA1* allele α-LELY. When in trans to an HE mutation this allele is expected to aggravate the phenotype to HPP due to a relative increase in the proportion of the defective protein (containing the HE mutation) incorporated into the membrane.[19,20] Although this patient is currently transfusion dependent, it is likely that impaired splenic function as a result of SCD (splenic autoinfarction) may improve her HPP-associated hemolysis.

The detection of HE mutations in patients with SCD is not surprising due to the positive selective pressure of malaria on both types of defects.[21,22] When patients with SCD present with a more severe phenotype than expected, it is important to consider any aspects of patient and family history that might suggest additional RBC abnormalities and to carefully examine peripheral blood smears for abnormal cells in addition to sickle cells. Ektacytometry is particularly useful when RBC cytoskeletal or hydration defects are suspected. Gene sequencing may be required to identify additional genetic abnormalities, especially when frequent transfusions obscure phenotypic RBC evaluation.

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## Abbreviations key

| | |
|---|---|
| **SCD** | sickle cell disease |
| **RBCs** | red blood cells |
| **ROS** | reactive oxygen species |
| **HE** | hereditary elliptocytosis |
| **HPP** | hereditary pyropoikilocytosis |
| **HU** | hydroxyurea |
| **TREAT** | Therapeutic Response Evaluation and Adherence Trial |
| **HbF** | hemoglobin F |
| **ARC** | absolute reticulocyte count |
| **MTD** | maximum tolerated dose |
| **EI** | elongation index |

| | |
|---|---|
| **HbD** | hemoglobin D |

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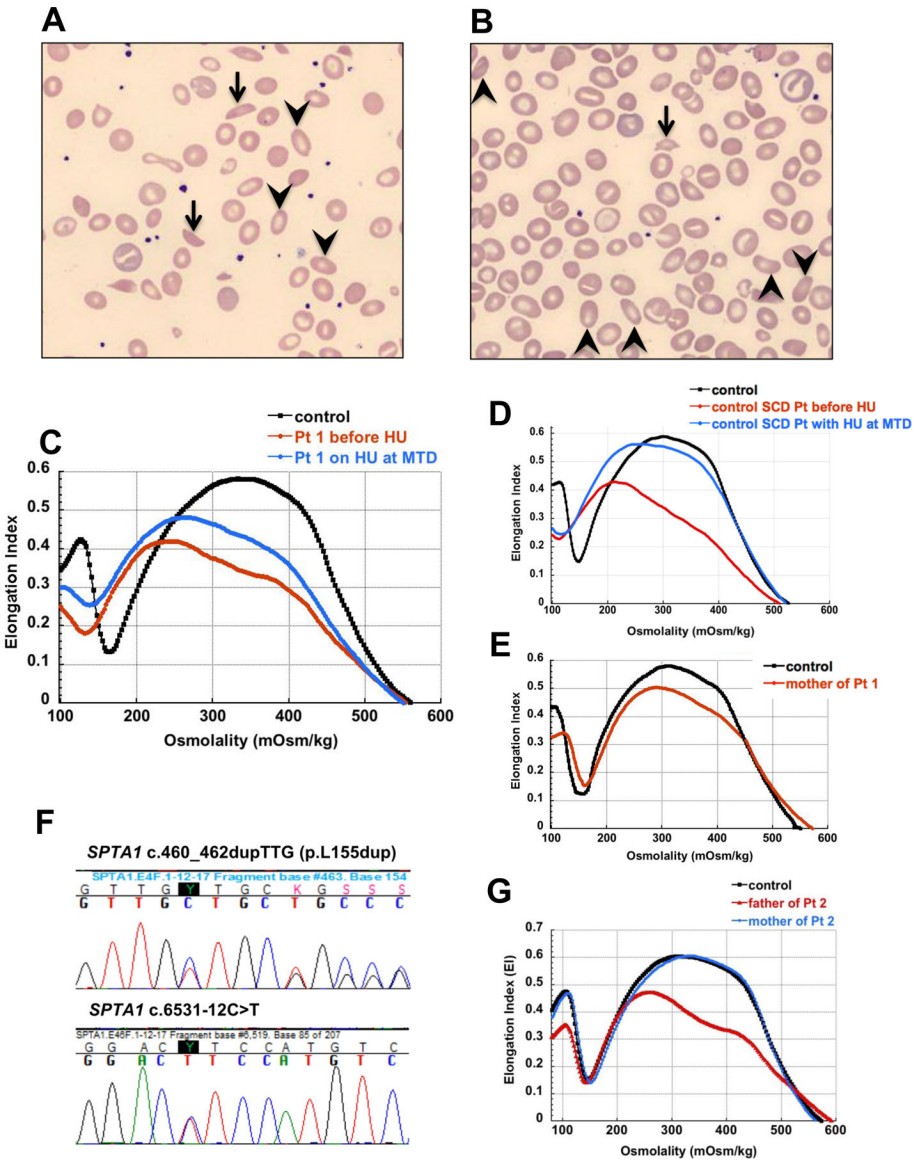


**FIGURE 1.**

(A & B) Wright stained peripheral blood smears from Patient 1, prior to treatment with HU (A) and after reaching MTD (B). Arrows indicate sickle cells; arrowheads indicate elliptocytes. (C) Ektacytometry (osmoscans) from Patient 1 performed before treatment with HU and after reaching MTD, showing only a modest improvement in deformability after treatment. This is in contrast to (D), osmoscans from a typical SCD patient before treatment with HU and after reaching MTD, showing a significant improvement in RBC deformability (as demonstrated by an increase in maximum Elongation Index) after treatment. (E) Ektacytometry of the mother of Patient 1, showing the typical trapezoidal curve of HE. Black curves in the ektacytometry plots represent tests of blood samples from healthy controls performed at the same time and under the same conditions as tests of the patients' samples. (F) DNA sequencing revealed the *SPTA1* mutation c.460_462dupTTG (p.L155dup) in Patient 1 and his mother and in Patient 2 and her father. In addition, the

*SPTA1* α-LELY variant [c.5572C>G (p.L1858V) and c.6531–12C>T] was found in Patient 2 and both her parents. (G) The ektacytometry curve of the father of Patient 2 is consistent with HPP, suggesting that the *SPTA1* HE mutation is in trans to the α-LELY variant in the father and Patient 2.

## TABLE 1

Sequencing results

| Subject | Gene | Allele | Mutation |
|---|---|---|---|
| Patient 1 | *SPTA1* | 1 | c.460 462dupTTG (p.L155dup) |
| | | 2 | no mutation identified |
| Mother of Patient 1 | *SPTA1* | 1 | c.460 462dupTTG (p.L155dup) |
| | | 2 | no mutation identified |
| Patient 2 | *SPTA1* | 1 | c.460 462dupTTG (p.L155dup) |
| | | 1 or 2 | c.5572C>G (p.L1858V) and c.6531–12C>T (alpha-LELY) |
| Mother of Patient 2 | *SPTA1* | 1 | c.5572C>G (p.L1858V) and c.6531–12C>T (alpha-LELY) |
| | | 2 | no mutation identified |
| Father of Patient 2 | *SPTA1* | 1 | c.460 462dupTTG (p.L155dup) |
| | | 1 or 2 | c.5572C>G (p.L1858V) and c.6531–12C>T (alpha-LELY) |