Novel exon 2 α spectrin mutation and intragenic crossover: three morphological phenotypes associated with four distinct α spectrin defects

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ABSTRACT

Hereditary pyropoikilocytosis is a severe hemolytic anemia caused by spectrin deficiency and defective spectrin dimer self-association, typically found in African populations. We describe two Utah families of northern European ancestry including 2 propositi with atypical non-microcytic hereditary pyropoikilocytosis, 7 hereditary elliptocytosis members and one asymptomatic carrier. The underlying molecular defect is a novel mutation in the alpha(α) spectrin gene, *SPTA*^{R34P} that impairs spectrin tetramer formation. It is inherited *in trans* to the hypomorphic *SPTA*$^{\alpha LELY}$ in the 2 propositi and 5 of 7 hereditary elliptocytosis individuals indicating that *SPTA*$^{\alpha LELY}$ is not the sole determinant of the variable clinical expression. α Spectrin mRNA was mildly decreased in all hereditary elliptocytosis subjects, whereas both hereditary pyropoikilocytosis propositi had a severe decrease to ~10% of normal. Genotyping identified a unique *SPTA* intragenic crossover and uniparental disomy in one hereditary elliptocytosis individual. Two additional crossover events demonstrated the susceptibility of *SPTA* gene to rearrangement and revealed a novel segregation of the two *SPTA*$^{\alpha LELY}$ mutations. We conclude that the profound phenotypic heterogeneity in these families can be attributed to the *SPTA*^{R34P} mutation in combination with: 1) inheritance *in trans* of either *SPTA*$^{\alpha LELY}$; or 2) the wild-type SPTA; 3) a decrease of α spectrin mRNA; and 4) *SPTA* intragenic crossover.

Introduction

Mutations of α spectrin (Sp) involving the Sp heterodimer self-association site (the αI domain of Sp) represent the most common group of erythrocyte membrane defects in hereditary elliptocytosis (HE) and a closely related disorder, hereditary pyropoikilocytosis (HPP; acronym HP also used).[1,2] HPP is characterized by extreme microcytosis with unique poikilocytic microspherocytic morphology; it is typically found among people of African origin and rarely among Caucasians.[3] The molecular defects underlying HPP are spectrin deficiency[4] and a severe Sp dimer (SpD) self-association defect,[5] which weaken the erythrocyte skeleton and cause membrane instability. Mutations that interfere with Sp tetramer (SpT) assembly typically occur in the αSp gene (*SPTA*) and HPP subjects are heterozygous, doubly heterozygous or homozygous for these mutations that cause structural defects in the protein.[1,6] In addition, HPP is associated with a second defect, which results in a decreased amount of the αSp peptide. Several molecular mechanisms have been described that underlie this partial Sp deficiency. These include defective αSp mRNA accumulation indicative of a malfunction in RNA processing:[7] increased degradation of αSp prior to incorporation into the membrane;[8] reduced levels of αSp mRNA and decreased synthesis of αSp protein;[8] and a splicing abnormality resulting in a premature stop codon.[9]

The clinical severity of HE/HPP is influenced by the precise location and type of the structural αSp mutation,[6] as well as by the inheritance of modifying alleles, such as the hypomorphic *SPTA*$^{\alpha LELY}$ polymorphism.[10] The *SPTA*$^{\alpha LELY}$ haplotype has two point mutations that are invariably linked: a C>G mutation in codon 1857 of exon 40 and a C>T mutation in intron 45 of the *SPTA* gene that is responsible for partial skipping of exon 46 in 50% of the αSp mRNA. The six amino acids encoded by exon 46 are essential for the functional assembly of α/β SpD resulting in a reduced amount of αSp peptide from this locus.[10] Inheritance of *SPTA*$^{\alpha LELY}$ *in cis* to a *SPTA*^{R28H} HE allele results in mild clinical symptoms,[11] whereas inheritance *in trans* causes a relative increase in the mRNA produced from the HE αSp allele, which exacerbates the SpD self-association defect and may lead to the HPP phenotype.[10] Thus the increased proportion of defective αβSpD causes a more severe morphological and clinical phenotype.

Here we describe two Utah families of northern European descent with a novel *SPTA*^{R34P} gene mutation interacting with four distinct αSp molecular configurations resulting in three phenotypes ranging from HPP to HE to normal morphology.

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Furthermore, the HPP phenotype is normocytic and not microcytic, and there are also numerous elliptocytes present on the peripheral smear; hence, we designate it as atypical HPP. In this manuscript, we clarify the molecular basis of these three phenotypes associated with the novel *SPTA*^{R34P} mutation and provide new insight into the complexity of erythrocyte membrane disorders.

Methods

Clinical and routine laboratory studies

All patients provided written, informed consent before participation in the study. The studies were approved by the University of Utah Institutional Review Board (IRB_00027669).

Family A

A 79-year old man (propositus of family A, A-I-3) (Figure 1A) presented with a life-long history of anemia and intermittent jaundice. He and his extended family trace their ancestry to Scottish and Scandinavian forebears who were among the first Mormon settlers of Utah Territory. He had a splenectomy two years prior to presentation that resulted in a partial amelioration of his anemia. He was aware that multiple family members had a history of abnormal red blood cell morphology and some were anemic. His erythrocyte morphology revealed anisopoikilocytosis, fragmented cells, microspherocytes, elliptocytosis and polychromasia (Figure 2A). Eleven first-degree relatives of his extended family over three generations were evaluated (Figure 1A).

Family B

An apparently unrelated 39-year old female (propositus of family B, B-I-1) (Figure 3A) was referred to one of the Authors (JTP) for evaluation of very high platelet count and suspected essential thrombocythemia. Since red cell fragmentation can be mistakenly reported as elevated platelet count by laboratory instruments, the accuracy of elevated platelet count has been verified by semi-quantitative estimation of platelet count by microscopic evaluation of blood smear by one of the Authors experienced with RBC fragmentation in several HPP patients identified over the last three decades (JTP). She had neonatal hyperbilirubinemia, and since birth has had episodes of jaundice with severe anemia (hemoglobin 70g/L). She had undergone splenectomy three years earlier, with improved anemia and no further episodes of jaundice. Her

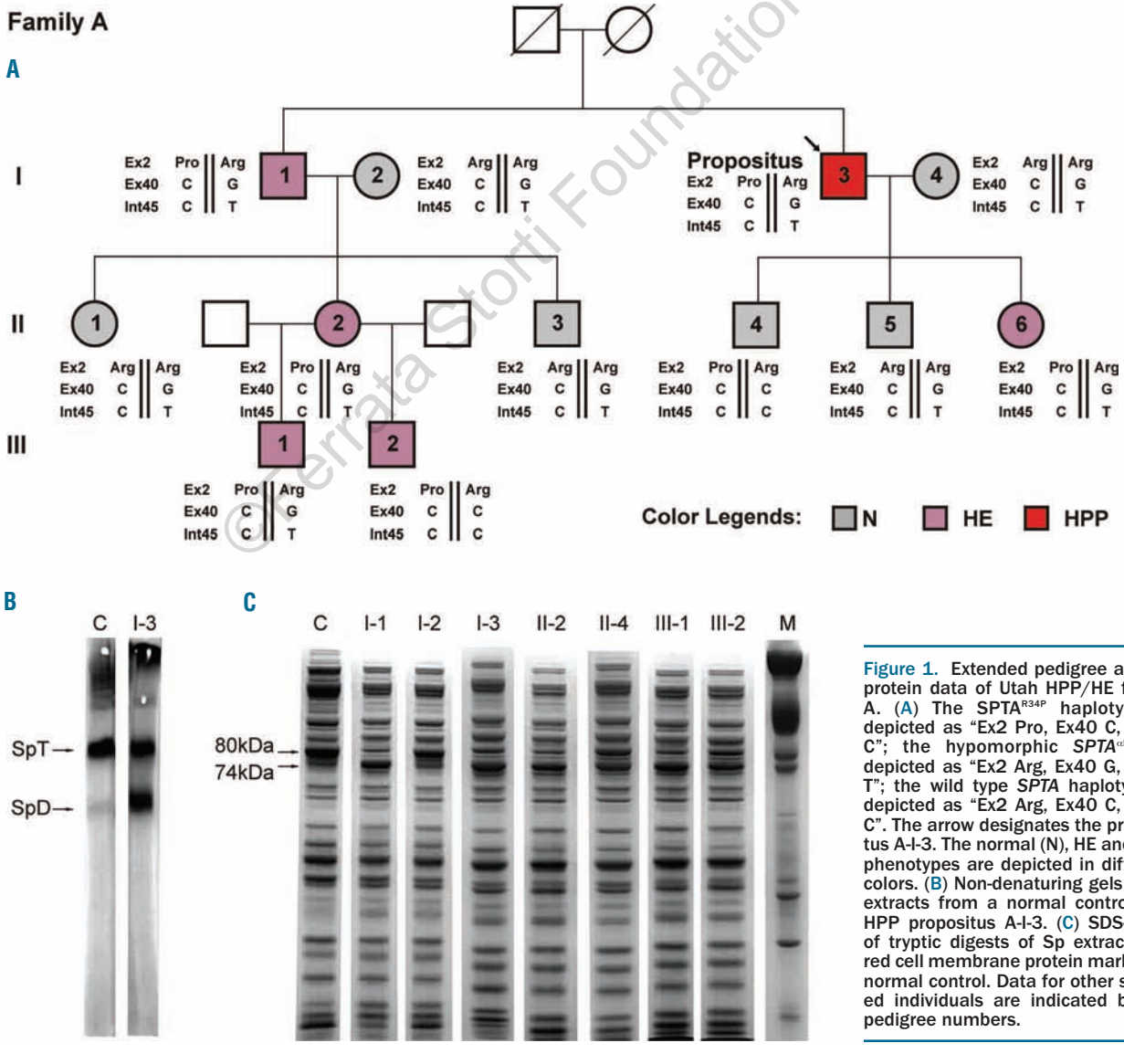

Figure 1. Extended pedigree and Sp protein data of Utah HPP/HE family A. (A) The SPTA^{R34P} haplotype is depicted as "Ex2 Pro, Ex40 C, Int45 C"; the hypomorphic *SPTA*$^{\alpha LELY}$ is depicted as "Ex2 Arg, Ex40 G, Int45 T"; the wild type *SPTA* haplotype is depicted as "Ex2 Arg, Ex40 C, Int45 C". The arrow designates the propositus A-I-3. The normal (N), HE and HPP phenotypes are depicted in different colors. (B) Non-denaturing gels of Sp extracts from a normal control and HPP propositus A-I-3. (C) SDS-PAGE of tryptic digests of Sp extracts. M: red cell membrane protein marker; C: normal control. Data for other selected individuals are indicated by the pedigree numbers.

ancestors were also among the first Mormon settlers of Utah Territory and were of north European ancestry. After splenectomy, she has had persistently elevated platelet counts (up to 1 million/μL), and was assumed to have essential thrombocythemia and treated intermittently with hydroxyurea. Her physical examination was unremarkable. Peripheral smear revealed significant anisopoikilocytosis, microspherocytes, elliptocytosis, and polychromasia (Figure 2B). Further studies revealed polyclonal hematopoiesis determined by the X-chromosome transcriptional assay[12] and the absence of *JAK2* and *cMPL* somatic mutations. The clinical diagnosis of atypical HPP and secondary thrombocytosis due to hemolytic anemia and splenectomy was made. Ten first-degree relatives of her extended family over two generations were evaluated (Figure 3A).

Age, gender, blood counts and erythrocyte morphology of all individuals who were studied from these two unrelated families are shown in the *Online Supplementary Table S1*.

Molecular and expression studies of the SPTA locus

Quantitation of the SPTA mRNA transcript

Reticulocyte SPTA mRNA was purified and levels were determined as described in the *Online Supplementary Methods*. [13,14]

SPTA locus genotyping

Genotyping of the *SPTA* locus was performed using the SNP markers, rs857677and rs2251969, flanking the $SPTA^{\alpha LELY}$ exon 40 and intron 45 mutations and two internal markers, rs3737515 and rs1616 (Applied Biosystems, CA, USA).

Erythrocyte membrane protein analysis

Membranes were prepared and the proteins subjected to SDS-PAGE and densitometric quantitation.[5] Details of this method are described in the *Online Supplementary Methods*.[15,16]

Comparative modeling of the spectrin tetramerization site

Details of this method are described in the *Online Supplementary Methods*.

Results

A novel $SPTA^{R34P}$ mutation

Sequencing of the *SPTA* gene revealed heterozygosity for a novel missense mutation at exon 2, codon 34: CGG>CCG, changing arginine to proline ($SPTA^{R34P}$; c101 G>C NM_003126), that was present in both propositi and some of their family members. This mutation was inherited *in trans* to the $SPTA^{\alpha LELY}$ hypomorphic allele in both propositi (A-I-3 and B-I-1) and in 5 individuals with an HE phenotype (A-I-1, A-II-2, A-II-6, A-III-1 and B-I-3). However, 2 HE individuals (A-III-2 and B-II-7) inherited the $SPTA^{R34P}$ mutation *in trans* to the wild-type *SPTA* allele. In addition, the latter genotype was also found in one individual (A-II-4) with no discernible red cell defect and normal blood counts (Figures 1A and 3A). These findings were confirmed on repeated testing.

Erythrocyte membrane skeleton analyses

Both propositi (A-I-3 and B-I-1) had a decreased amount of Sp, based on densitometry of SDS polyacrylamide gels and a comparison of the spectrin/band 3 ratios, consistent with their HPP phenotype. Functional and structural analyses of Sp from 7 affected individuals (2 HPP and 5 HE; no data on A-II-6 and B-II-7) in both families revealed an increase in SpD and a corresponding decrease in SpT (Figures 1B and 3B; Table 1A and B). This functional Sp tetramerization abnormality was due to a structural defect causing an elevated amount of a mutant 74kDa SpαI peptide after limited tryptic digestion and a concomitant reduction in the normal 80kDa SpαI peptide (Figures 1C and 3C; Table 1A and B). There was a significant correlation between abnormal morphology, levels of SpD, and the proportion of 74kDa SpαI mutant peptide. One exception was A-II-4, who had normal morphology, despite increased amounts of SpD and 74kDa SpαI peptide, similar to A-III-2, who presented with HE (Table 1A and B). Eleven normal family members from the two pedigrees had normal SpD values and a normal SpαI domain.

Model of the Sp tetramerization site

A computer model was constructed showing the influence of the $SPTA^{R34P}$ mutation on the Sp tetramerization site (Figure 4). Arginine 34 is situated in helix C of the partial αSp repeat 0 and participates in electrostatic interactions with glutamic acid residues 2022 and 2029 in helix A of the partial repeat 17 of βSp (Figure 4A and C). Arginine 34 also forms a hydrogen bond with glutamic acid 2029, and the aliphatic portion of the amino acid can participate

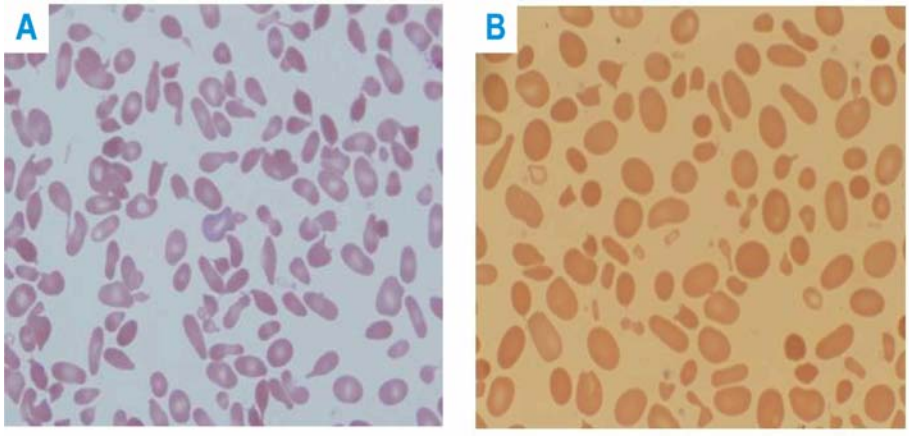

Figure 2. Red cell morphological features of the two HPP propositi. Peripheral blood smear of propositus A-I-3 (left) and propositus B-I-1 (right). Both smears show marked anisopoikilocytosis, elliptocytes, microspherocytes, ovalocytes, and red blood cells with fragments and bizarre shapes.

in hydrophobic interactions at the Sp tetramerisation site (Figure 4E). The proline 34 mutation creates a cavity, which abolishes the electrostatic interactions and also disrupts the hydrogen bond and van der Waal's forces (Figure 4B, D and F). The conformation of helix C of the partial αSp repeat 0 is altered (Figure 4G) and the helical propensity of the polypeptide is decreased (*Online Supplementary Table S3*).

Quantitation of αSp mRNA

The effect of the *SPTA*$^{\alpha LELY}$ allele is to reduce the amount of Sp peptide from this locus. Inheritance of *SPTA*$^{\alpha LELY}$ *in trans* to an HE allele results in a more severe phenotype by increasing the relative quantity of defective Sp peptide by a posttranslational mechanism. All subjects who inherited the *SPTA*^{R34P} mutation *in trans* to *SPTA*$^{\alpha LELY}$ had a greater level of mutant exon 2 mRNA compared to wild type at a ratio approximating 3:1 (Table 1A and B; Figures 1 and 2). Unexpectedly, however, a similar ratio was obtained in the 3 subjects (A-II-4, A-III-2 and B-II-7) with *SPTA*^{R34P} but lacking *SPTA*$^{\alpha LELY}$.

The amount of total SPTA transcript produced in reticulocytes was also measured and this showed that in all HE subjects the level of SPTA mRNA was decreased to ~85% of controls, whereas in all individuals with normal morphology the expression level was unaffected (Table 1A and B). Both HPP subjects had markedly decreased levels of total SPTA reticulocyte mRNA (~10% of normal).

Molecular studies of the SPTA locus

Incongruous results were obtained for individual B-II-7 and his mother, B-I-3, who both had an HE phenotype and were heterozygous for the *SPTA*^{R34P} mutation. The mother had the *SPTA*$^{\alpha LELY}$ polymorphism on her normal (R34) allele and since her son did not have this allele, he presumably inherited the wild-type allele from his father (not available for study). His mother would thus have passed on the mutant *SPTA*^{R34P} allele to him. However, the haplotype of this *SPTA*^{R34P} allele was different to that of his mother and required further investigation (Figure 3A).

Intragenic crossover and uniparental disomy

Informative SNP analysis was performed on the *SPTA* locus of subject B-II-7 to establish his haplotype and to compare it to the haplotype of his mother (B-I-3) and 2 available siblings (the father's DNA was unavailable). An intragenic crossover was found, which resulted in loss of heterozygosity (LOH) most likely from uniparental disomy (UPD) and homozygosity for the wild-type exon 40 (C) and intron 45 (C) allele without the *SPTA*$^{\alpha LELY}$ mutations present in his mother (Figure 5). We could not formally exclude the possibility that this observed LOH was not due to LOH from deletion; unfortunately this could not be formally tested because of the limited amount of DNA available.

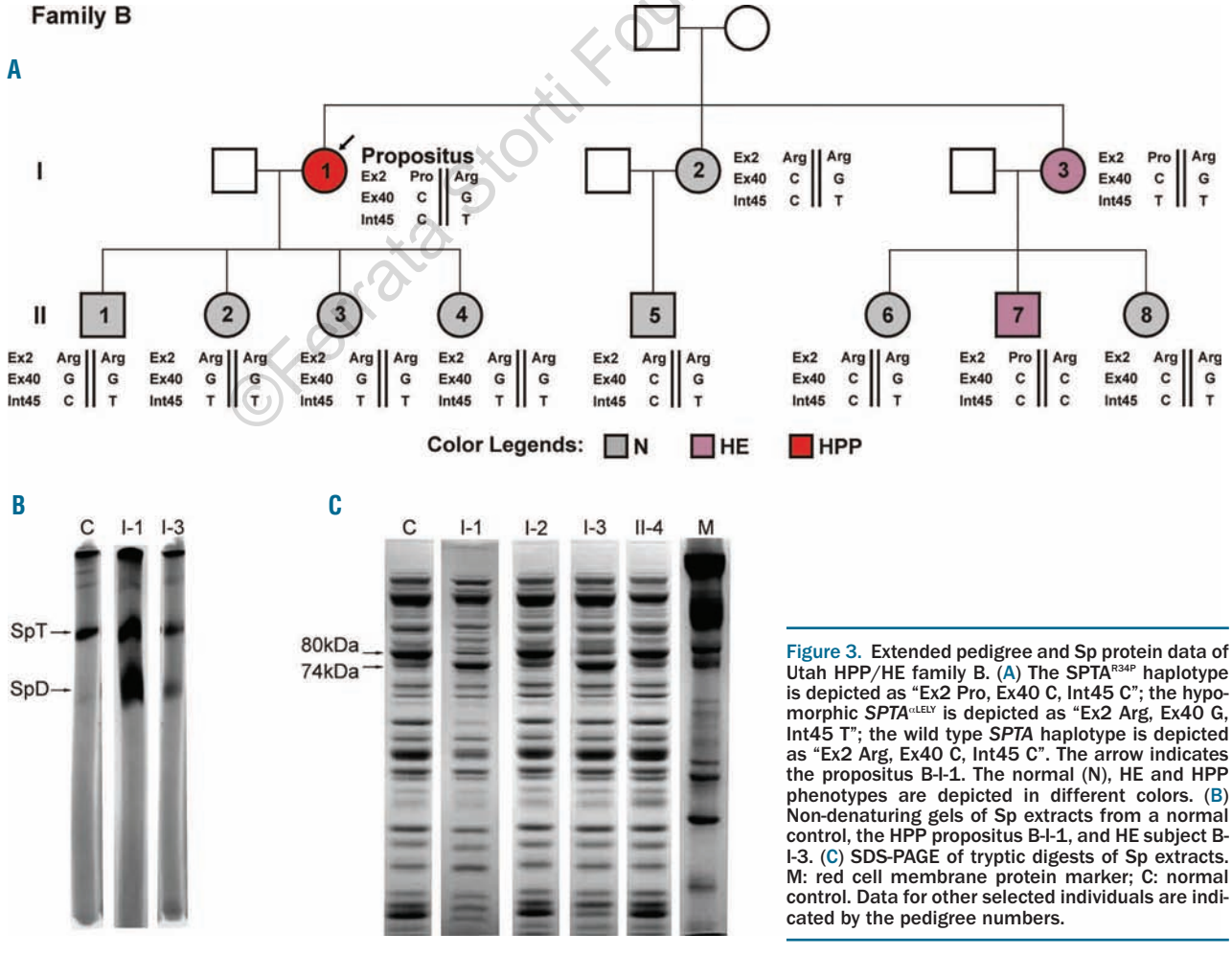

Figure 3. Extended pedigree and Sp protein data of Utah HPP/HE family B. (A) The SPTA^{R34P} haplotype is depicted as "Ex2 Pro, Ex40 C, Int45 C"; the hypomorphic *SPTA*$^{\alpha LELY}$ is depicted as "Ex2 Arg, Ex40 G, Int45 T"; the wild type *SPTA* haplotype is depicted as "Ex2 Arg, Ex40 C, Int45 C". The arrow indicates the propositus B-I-1. The normal (N), HE and HPP phenotypes are depicted in different colors. (B) Non-denaturing gels of Sp extracts from a normal control, the HPP propositus B-I-1, and HE subject B-I-3. (C) SDS-PAGE of tryptic digests of Sp extracts. M: red cell membrane protein marker; C: normal control. Data for other selected individuals are indicated by the pedigree numbers.

Segregation of the SPTA$^{\alpha LELY}$ mutations

The haplotype of individual B-I-3 further revealed a novel segregation of the two *SPTA*$^{\alpha LELY}$ mutations since she had wild-type exon 40 (C), but mutant intron 45 (T). Her nephew, B-II-1, also showed separation of the two mutations, but in this case he had the exon 40 mutation (G) and wild-type intron 45 (C) (Figure 2A). This is the first reported instance where these two mutations have not been inherited together and presumably reflects additional examples of intragenic crossover of this large gene.

Discussion

Hereditary pyropoikilocytosis has a striking morphological phenotype that, although very rare, has been of intense interest to hematologists. Its characteristic features are severe microcytosis and spherocytes with unusual poikilocytic projections. While it is almost always found in Black populations, and occasionally in people of Mediterranean origin, it has not been described among North Europeans. We have previously reported that, in some families, the HPP abnormality clusters with relatives who either have HE or who exhibit a normal hematologic phenotype.[5,17] However, a detailed analysis of the erythrocyte membrane skeleton revealed subtle functional and structural defects in Sp in those individuals with normal hematologic indices, indicating that they are asymptomatic carriers.[5,17]

The molecular basis of the diverse phenotypes present in a single family is still poorly understood. Most of the mutations associated with the HE/HPP phenotype are missense mutations, generally in exon 2 of the *SPTA* gene, which impair the formation of αβSp heterotetramers. However, for the HPP phenotype, an additional hypomorphic mutation is thought to be necessary, which results in partial spectrin deficiency.

The SPTA^{R34P} mutation perturbs the Sp tetramerization site

The two HPP/HE families of northern European descent we describe here have a novel exon 2 *SPTA*^{R34P} mutation. Arginine 34 is located in αSp repeat 0 and it interacts with key residues on βSp repeat 17 to stabilize the interface of the hybrid SpT.[18,19] Mutation of this charged, basic residue to a compact, uncharged and cyclic proline, reduces the helical content of the repeat (*Online Supplementary Table S3*) and perturbs the interaction between Sp heterodimers as shown by molecular modeling (Figure 4).

Numerous missense mutations have been described in αSp repeat 0, but none involve proline. In contrast, the majority of pathogenic mutations in the helical linker regions between αSp repeats are due to proline substitutions, which cause unfolding of the links and destabilization of the adjacent repeats.[20] These long-range effects on SpT formation imply that the structural consequences of

Table 1. αSp protein and mRNA expression data for Families A and B.

Table 1A. Family A

Pedigree number	I-1	I-2	I-3	I-4	II-1	II-2	II-3	II-4	II-5	II-6	III-1	III-2
Age (years)	83	--	79	--	56	52	47	48	42	41	20	11
Sp dimers	51%	5%	49%	--	16%	44%	16%	23%	--	--	40%	25%
SpαI 80kDa peptide	22%	62%	30%	--	60%	25%	58%	45%	--	--	27%	46%
SpαI 74kDa peptide	78%	38%	70%	--	40%	75%	42%	55%	--	--	73%	54%
Quantitative αSp mRNA ratio*	29/71	100/0	20/80	100/0	100/0	24/76	100/0	23/77	100/0	20/80	25/75	28/72
Reticulocyte αSp mRNA expression	85%	100%	10%	100%	100%	85%	100%	100%	100%	85%	85%	85%
Erythrocyte morphology	HE	N	HPP	N	N	HE	N	N	N	HE	HE	HE
Genotype	*SPTA*^{R34P}/ *SPTA*$^{\alpha LELY}$	*SPTA*/ *SPTA*	*SPTA*^{R34P}/ *SPTA*$^{\alpha LELY}$	*SPTA*/ *SPTA*	*SPTA*/ *SPTA*	*SPTA*^{R34P}/ *SPTA*$^{\alpha LELY}$	*SPTA*/ *SPTA*	*SPTA*^{R34P}/ *SPTA*	*SPTA*/ *SPTA*	*SPTA*^{R34P}/ *SPTA*$^{\alpha LELY}$	*SPTA*^{R34P}/ *SPTA*$^{\alpha LELY}$	*SPTA*^{R34P}/ *SPTA*

**Quantitative αSp mRNA ratio of R34 wild-type allele/P34 mutant allele.*

Table 1B. Family B

Pedigree number	I-1	I-2	I-3	II-1	II-2	II-3	II-4	II-5	II-6	II-7	II-8
Age (years)	39	38	35	17	12	9	8	8	10	7	4
Sp dimers	56%	N	40%	N	N	N	N	N	N	--	N
SpαI 80kDa peptide	21%	65%	32%	65%	65%	63%	65%	65%	68%	--	69%
SpαI 74kDa peptide	79%	35%	68%	35%	35%	37%	35%	35%	32%	--	31%
Quantitative αSp mRNA ratio*	20/80	100/0	20/80	100/0	100/0	100/0	100/0	100/0	100/0	23/77	100/0
Reticulocyte αSp mRNA expression	10%	100%	85%	100%	100%	100%	100%	100%	100%	85%	100%
Erythrocyte morphology	HPP	N	HE	N	N	N	N	N	N	HE	N
Genotype	*SPTA*^{R34P}/ *SPTA*$^{\alpha LELY}$	*SPTA*/ *SPTA*	*SPTA*$^{R34P, int45LELY}$/ *SPTA*$^{\alpha LELY}$ *SPTA*	*SPTA*/ *SPTA*	*SPTA*/ *SPTA*	*SPTA*/ *SPTA*	*SPTA*/ *SPTA*	*SPTA*/ *SPTA*	*SPTA*/ *SPTA*	*SPTA*^{R34P}/ *SPTA*	*SPTA*/ *SPTA*

**Quantitative αSp mRNA ratio of R34 wild-type allele/P34 mutant allele.*

helix-breaking proline mutations are quite severe. In the families reported here, the effect of the *SPTA*^{R34P} mutation is relatively mild, causing an increase in SpD of only ~25%, when inherited without other modifying alleles (A-II-4). This biochemical defect is insufficient to affect erythrocyte morphology and subject A-II-4 is an asymptomatic carrier with a normal hematologic phenotype.

One other pathogenic mutation has been described at amino acid 34 of αSp (*SPTA*^{R34W}), which caused mild elliptocytosis in a single family.[21] The HE proband inherited *SPTA*^{R34W} *in trans* to *SPTA*$^{\alpha LELY}$, whereas his father, who also had the *SPTA*^{R34W} mutation, did not have *SPTA*$^{\alpha LELY}$ and showed normal morphology, despite decreased Sp tetramer formation. These two arginine 34 mutations (R>W and R>P) therefore have similar and relatively mild effects. Further *in vitro* binding studies and crystal structure analysis of the tryptophan 34 mutant revealed a slight structural destabilization of the Sp monomer, but there was no effect on the binding of the mutant αSp0 peptide to βSp16-17 peptide when compared to wild type.[19,22] Evolutionary sequence analysis revealed that arginine 34 is a highly conserved residue, although for some species diversity exists; for example, glutamine is present in rat and mouse αSp. These combined data support the findings in this study, which indicate that even though the *SPTA*^{R34P} mutation perturbs SpT formation, it is not severe enough to cause clinical symptoms in the absence of hypomorphic modifier alleles.

At present, we do not have any data about the frequency of this novel *SPTA*^{R34P} mutation in the Utah population. The propositi of both currently studied families, although it is not known whether they are related, have ancestry originating among the early Mormon settlers in Utah. As a large proportion of the Utah population trace their ancestry from these early Mormons, our report of affected individuals may indicate that the *SPTA*^{R34P} mutation represents a *founder effect* in this Mormon community.

Intragenic Sp crossover

An additional novel finding in one of the HE subjects, B-II-7, was an intragenic crossover manifesting as an LOH, most likely generated by UPD in the *SPTA* gene in the region between exon 40 and intron 45. We acknowledge that this observed LOH could not formally exclude a deletion as a mechanism of LOH; however, this deletion would likely result in a more pronounced RBC membrane disarray than only HE seen in our patient. In point of fact, a recent report[23] indicated that such a large deletion would result in a striking morphological abnormality. This LOH resulted in the loss of the *SPTA*$^{\alpha LELY}$ allele, but the exon 2 *SPTA*^{R34P} mutation was unaffected. To further characterize this UPD, presumably originating from the father's *SPTA* gene (father's DNA not available), haplotype analysis was performed using additional polymorphic markers, defining this *SPTA* UPD as "BCAC", which replaces the "ACAT" inherited from the mother (Figure 5).

Two further apparent examples of intragenic crossover that possibly occurred in the ancestry of individuals B-I-3 and B-II-1 demonstrate the susceptibility of the *SPTA* gene to intragenic recombination, likely due to the large size of

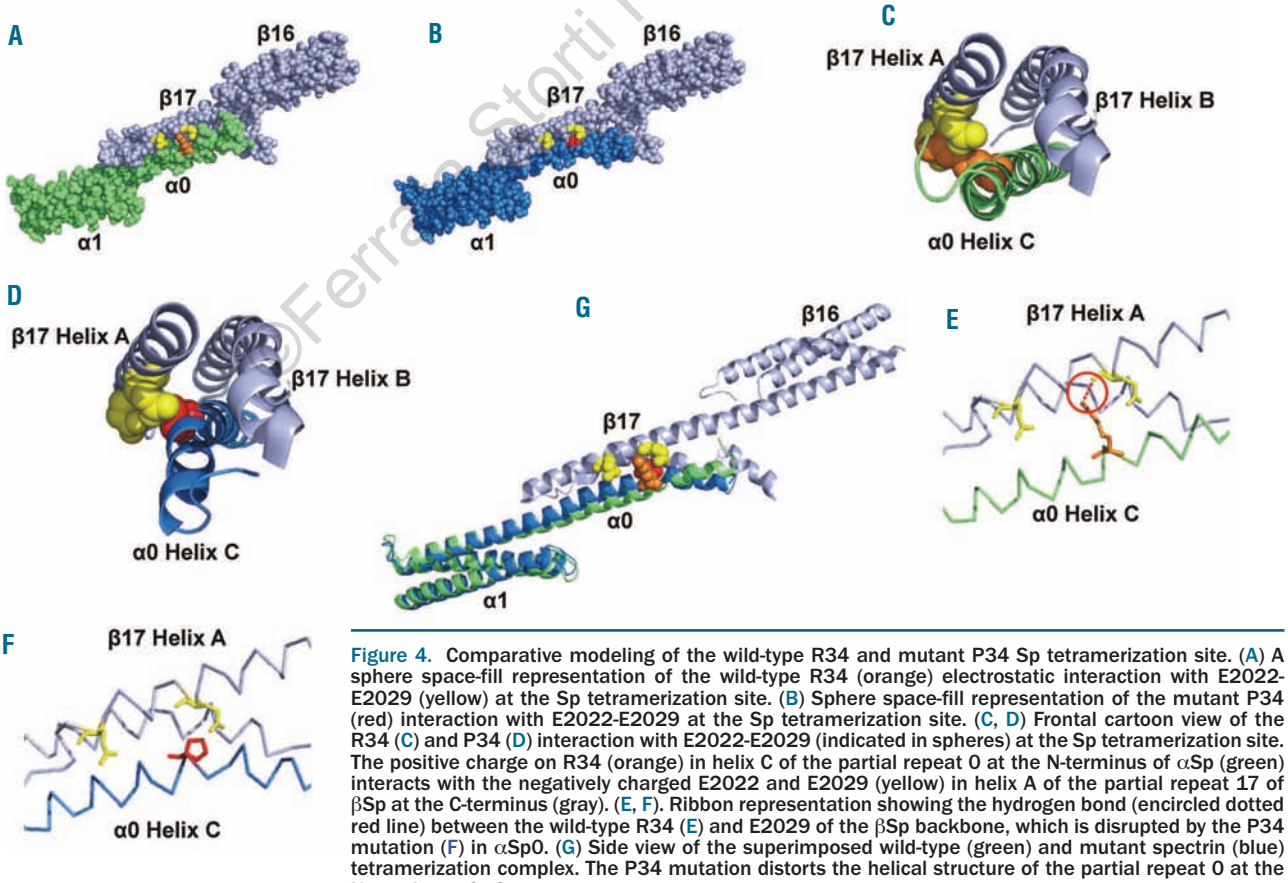

Figure 4. Comparative modeling of the wild-type R34 and mutant P34 Sp tetramerization site. (A) A sphere space-fill representation of the wild-type R34 (orange) electrostatic interaction with E2022-E2029 (yellow) at the Sp tetramerization site. (B) Sphere space-fill representation of the mutant P34 (red) interaction with E2022-E2029 at the Sp tetramerization site. (C, D) Frontal cartoon view of the R34 (C) and P34 (D) interaction with E2022-E2029 (indicated in spheres) at the Sp tetramerization site. The positive charge on R34 (orange) in helix C of the partial repeat 0 at the N-terminus of αSp (green) interacts with the negatively charged E2022 and E2029 (yellow) in helix A of the partial repeat 17 of βSp at the C-terminus (gray). (E, F). Ribbon representation showing the hydrogen bond (encircled dotted red line) between the wild-type R34 (E) and E2029 of the βSp backbone, which is disrupted by the P34 mutation (F) in αSp0. (G) Side view of the superimposed wild-type (green) and mutant spectrin (blue) tetramerization complex. The P34 mutation distorts the helical structure of the partial repeat 0 at the N-terminus of αSp.

this gene and its numerous homologous repeats. These crossovers also represent the first report where the exon 40 and intron 45 mutations, characteristic of *SPTA*$^{\alpha LELY}$, have been separated. To date, these have always occurred on the same allele so that the *SPTA*$^{\alpha LELY}$ genotype has been defined as GT, in contrast to wild-type CC.[10,24] In subjects B-I-3 and B-II-1, the genotype has changed to CT and GC, respectively (Figure 3A). The exon 40 (C→G) mutation changes a leucine to valine, which alters the αSp tryptic digest pattern, whereas the intron 45 (C→T) mutation causes skipping of exon 46 and prevents formation of αβSp heterodimers, resulting in low expression of the SpαLELY peptide. Subject B-I-3 has the *SPTA*$^{\alpha LELY}$ allele *in trans* to the *SPTA*^{R34P} mutation, but has also gained the intron 45 mutation *in cis* to the codon 34 mutation, so both alleles will be produced in reduced amounts.

Decreased expression of αSp mRNA in reticulocytes

All 10 subjects who were heterozygous for the *SPTA*^{R34P} mutation have unequal levels of αSp mRNA from the two alleles, with the P34 mutant allele accounting for ~70% of the αSp mRNA in reticulocytes. Seven of these subjects inherited the hypomorphic *SPTA*$^{\alpha LELY}$ *in trans*, which would result in a relative increase in the mutant Sp. However, 3 of the subjects (A-II-4, A-III-2 and B-II-7) did not have the *SPTA*$^{\alpha LELY}$ polymorphism and the molecular basis of the unexpected reduced proportion of αSp transcripts from the wild-type allele is currently unknown. These anomalies highlight our limited knowledge of modifying factors that influence the transcription and translation of αSp.

Quantitation of the total amount of reticulocyte αSp mRNA revealed normal amounts in the asymptomatic carrier, but this was reduced to ~85% for all HE subjects. Since αSp peptides are produced in a 2- to 3-fold excess over βSp[8], the rate-limiting step in αβSp heterodimer formation is the synthesis of βSp, and a slight reduction in αSp mRNA should not have an effect on the Sp content of the erythrocyte membrane. For the 2 HPP propositi, however, only 10% αSp mRNA was present in reticulocytes and this resulted in a partial deficiency of Sp. However, we suggest that this transcriptional defect of αSp gene is a pivotal contribution to the described phenotype, thus the accuracy of this important observation has been confirmed several times using separately obtained blood specimens. Currently the molecular basis of this phenomenon remains unexplained. It may be due to mutation in the promoter region of this gene (not detected) or it also may be due to aberrant regulatory molecules controlling αSp transcription, such as microRNA or other regulatory noncoding RNAs, or an abnormal transcription factor, such as that described with a GATA1 mutation causing multiple phenotypes.[25] Unfortunately, fresh blood samples from these propositi are no longer available for studies designed to address these possibilities.

Genotype – phenotype correlations

The novel *SPTA*^{R34P} mutation in the two Utah families reported here is associated with unexpected genotypic and phenotypic heterogeneity (Table 2). Three distinct phenotypes of atypical HPP, HE, and an asymptomatic carrier state with normal erythrocyte morphology were

Table 2. Genotype-phenotype correlation

Genotype	αSp mRNA	Phenotype	Subjects
SPTA^{R34P}/*SPTA*$^{\alpha LELY}$	10%	Atypical HPP	AI-3, BI-1
SPTA^{R34P}/*SPTA*$^{\alpha LELY}$	85%	HE	AI-1, AII-2, AII-6, AIII-1
SPTA^{R34P}/*SPTA*	85%	HE	AIII-2, BII-7
SPTA^{R34P}/*SPTA*	100%	Normal	AII-4
SPTA$^{R34P, int45LELY}$/*SPTA*$^{\alpha LELY}$	85%	HE	BI-3

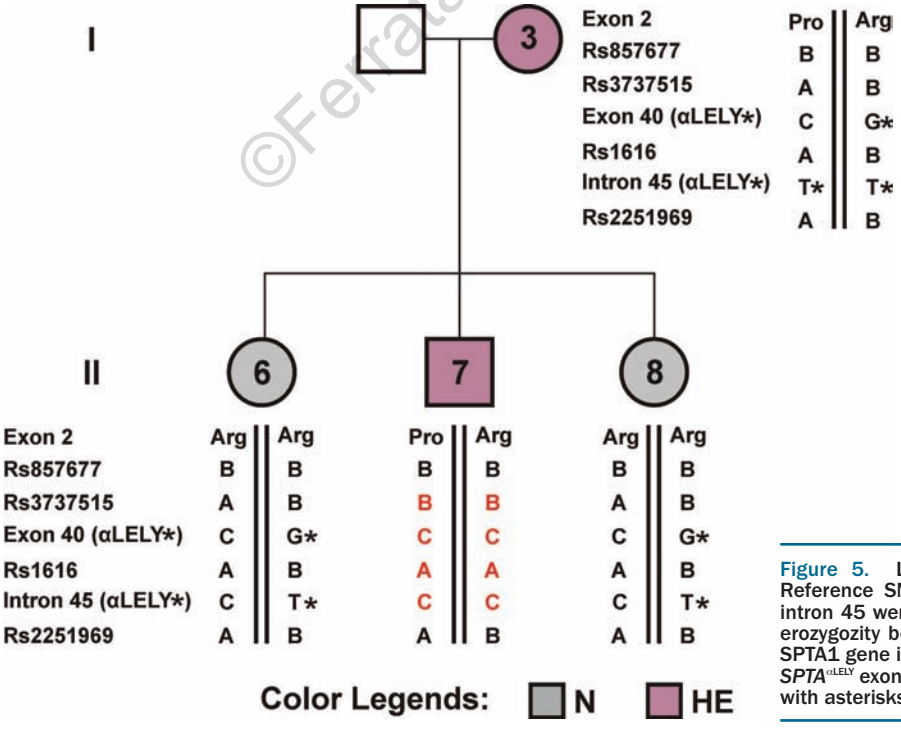

Figure 5. Loss of heterozygozity in *SPTA1* gene. Reference SNPs and the sequence of exon 40 and intron 45 were used to show the region of loss of heterozygozity between rs857677 and rs2251969 of the SPTA1 gene in individual B-II-7 (red) from family B. The *SPTA*$^{\alpha LELY}$ exon 40 and intron 45 mutations are indicated with asterisks.

observed. The HPP propositi exhibited the typical spherocytic poikilocytosis diagnostic of this disorder, but elliptocytes were also present on the peripheral blood smears, and unlike other HPP subjects, they were not microcytic. Biochemically they showed the characteristic protein defects of markedly reduced αβSp heterodimer self-association, a reduced proportion of Sp oligomers, an abnormal tryptic digest pattern, and partial Sp deficiency.

The clinical expression of an elliptocytogenic αSp mutation is influenced by the co-inheritance of hypomorphic alleles such as *SPTA*$^{\alpha LELY}$, which exacerbates the clinical phenotype, when inherited *in trans* to the mutant allele, as evidenced in the 2 propositi and HE subjects AI-1, AII-2, AII-6 and AIII-1 (Table 2). However, HE subjects A-III-2 and B-II-7 lacked the *SPTA*$^{\alpha LELY}$ polymorphism, but were clinically similarly affected. In addition, subject B-I-3 was homozygous for the intron 45 mutation, which would cause low expression of both mutant and wild-type alleles and thus eliminate the effect. This study demonstrates that in these two families *SPTA*$^{\alpha LELY}$ is not the only modifying allele influencing hematologic parameters. A key additional factor modulating clinical presentation is the level of total αSp mRNA in reticulocytes, which correlates with the phenotype (Table 2).

In conclusion, in these two Utah families a novel *SPTA*^{R34P} mutation is present in combination with four distinct genetic/biochemical defects: 1) inheritance *in trans* of either the hypomorphic *SPTA*$^{\alpha LELY}$; or 2) the wild-type *SPTA* allele; 3) an as yet unexplained decrease in total αSp transcripts; and 4) a unique intragenic crossover (Table 2). These different interactions between the *SPTA* locus and modifying alleles/factors provide the basis for all three distinct morphological phenotypes and offer an illuminating example of unanticipated complexity of inherited hematologic disorders that stem from a single gene locus.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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