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ORIGINAL ARTICLE

Molecular insights into hereditary elliptocytosis and pyropoikilocytosis: NGS uncovers multiple potential candidate genes

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Abstract

Hereditary elliptocytosis (HE) and pyropoikilocytosis (HPP) are considered a group of hemolytic anemias (HE/HPP) due to inherited abnormalities of erythrocyte membrane proteins with a worldwide distribution. Most cases are associated with molecular abnormalities linked to spectrin, band 4.1, and ankyrin. The present study aimed to identify significant molecular signatures on a target panel of 8 genes using whole exome sequencing (WES) in 9 Bahraini patients with elliptocytosis. Case selection was based on presence of anemia not associated with iron deficiency or hemoglobinopathy and demonstrating > 50% elliptocytes in blood smears. The c.779 T > C mutation of SPTA1 (Spectrin alpha), which is a known deleterious missense mutation that inhibits normal association of spectrin molecules to form tetramers, was seen in 4 patients in homozygous ($n = 1$) and heterozygous ($n = 3$) states. The αLELY abnormality in association with compound heterozygous mutations in SPTA1 was present in 5 patients (2 associated with the SPTA1 c.779 T > C variant; 3 with c.3487 T > G and various other SPTA1 mutations of uncertain/unknown significance). Seven patients had SPTB (Spectrin beta) mutations, predicted as likely benign by in silico analysis. A novel EPB41 (Erythrocyte Membrane Protein Band 4.1) mutation with potential deleterious impact was also seen. Finally, 2 cases showed an InDel (insertion–deletion mutations) abnormality in the gene that codes for the mechanosensitive ion-channel PIEZO (Piezo Type Mechanosensitive Ion Channel Component 1). PIEZO mutations are reported to cause red cell dehydration but have not been previously described in HE/HPP. Results of this study confirm the involvement of previously reported abnormalities in SPTA1 and suggest possible involvement of other candidate genes in a disorder involving polygenic interactions.

Keywords Hereditary elliptocytosis · Pyropoikilocytosis · Hemolytic anemia · RBC dehydration

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Introduction

Hereditary elliptocytosis (HE) is a type of hemolytic anemia caused by inherited defects in erythrocyte membrane proteins that is characterized by the presence of elliptical red cells in peripheral blood smears of patients. Hereditary pyropoikilocytosis (HPP) is part of the clinical-morphological spectrum of HE (HE/HPP) characterized by moderate to severe hemolytic anemia and elliptocytosis with poikilocytosis (fragmented cells, microspherocytosis). Most cases have been associated with molecular abnormalities linked to membrane proteins spectrin, band 4.1, and ankyrin.

The major components of the erythrocyte membrane are (i) a surface phospholipid bilayer that has integral membrane proteins (Band 3, glycophorin) embedded within it, (ii) an underlying cytoskeleton composed of a meshwork of peripheral membrane proteins (α-spectrin, β-spectrin), and (iii) linker protein-complexes (4.1R-, 4.2- and ankyrin-based anchorage complexes) that tether the relatively fluid lipid-bilayer to the cytoskeleton [1]. This arrangement not only stabilizes the surface membrane but also confers the red cell its special structure-functional characteristics: biconcave shape, deformability, and normal lifespan [2].

Mutations involving one or more membrane proteins can significantly impact the horizontal or vertical interactions between these proteins and thereby affect the stability of the erythrocyte membrane. This manifests as decreased red cell deformability and reduced red cell circulation half-life due to premature destruction of erythrocytes. Ion channels, transporters, and pumps on the lipid bilayer of the red cell membrane also influence cell deformability by regulating the ion balance and volume of the cell. Among ion channels, PIEZO1 (a mechanosensitive non-selective cation channel) regulates Ca^{2+} influx and thereby maintains red cell volume homeostasis. The Ca^{2+-}activated K^+ channel (Gardos channel), $Cl^-/HCO3^-$ antiporter Band 3, and the plasma membrane Ca^{2+} ATPase pump (PMCA) are essential for red cell homeostasis [2–4]. Finally, erythrocyte deformability also depends on ATP content and antioxidant systems [5].

Hereditary elliptocytosis (HE) has a global distribution with a high incidence of 1 in 200 reported from Africa. However, most cases are asymptomatic and so its true prevalence is largely underestimated [2]. The membrane defect underlying HE is due to weakening of horizontal linkages involving spectrin-spectrin and/or spectrin-protein 4.1R junctional interactions. The resultant membrane instability leads to progressive transformation of shape from discocyte to elliptocyte due to membrane loss with decreased surface area, and red cell fragmentation in those most severely affected [6].

Elliptocytosis has been shown to result from autosomal dominant mutations in the α-spectrin gene (SPTA1), β-spectrin gene (SPTB), and the EPB41 gene encoding protein 4.1R [6]. Mutations in *SPTA1* are most frequent and could account for about 65% of HE cases, followed by *SPTB in* 30% and EPB41 in ~ 5% of cases [7, 8]. Inheritance of a homozygous SPTA1 mutation or of compound heterozygous mutations can lead to the more severe clinical phenotype of hereditary pyropoikilocytosis (HPP) that is characterized by an overt hemolytic anemia [9].

A common polymorphic low-expression allele of spectrin (α^{LELY}) can give rise to a more severe clinical phenotype when it is inherited on the alternate allele in trans with a heterozygous mutation in α-spectrin [10–12]. The α^{LELY} allele results from a SNP within intron 45 (c.6531-12C > T) along with a missense mutation in exon 40 (c.5572C > G) resulting in amino acid substitution of leucine with valine (p.L1858V) on the same allele [12, 13]. The closeness of the intronic SNP to the exon splice site leads to partial skipping of exon 46 in about 50% of mRNA transcripts from the α^{LELY} allele. Dimerization of the α-spectrin protein from the α^{LELY} allele is impacted [14]. Generally, spectrin is produced in 3–fourfold excess of the amount required; hence, this polymorphism is mostly silent unless it is inherited with other deleterious mutation(s) [12–15].

The advent of next-generation sequencing platforms provides a rapid approach to the molecular diagnosis of the condition by screening for possible molecular abnormalities in several candidate genes involving erythrocyte membrane proteins, but few studies have been reported in the literature.

Methods

Peripheral blood smears of patients that were submitted to the hematology laboratories of participating hospitals (Salmaniya Medical Complex, SMC and King Hamad University hospital, KHUH) for the investigation of anemia, were screened for the presence of elliptocytosis. The criteria for diagnosis of HE were as follows: (a) > 10% elliptocytes in peripheral blood smear and (b) exclusion of iron, vitamin B_{12} and folate deficiencies, thalassemia and myelodysplastic syndrome [16]. For molecular genetic studies, DNA was extracted from 9 samples that showed > 50% elliptocytes, compatible with the morphologic diagnosis of severe HE, and processed for next-generation sequencing analysis. The results of routinely done laboratory tests including blood counts and biochemistry (serum bilirubin and lactate dehydrogenase, LDH) were documented. The study was approved by the Research and Research Ethics committees of the College of Medicine and Medical Sciences of Arabian Gulf University, Ministry of Health, SMC and KHUH prior to obtaining the samples from the patients.

Whole exome sequencing

Eight genes with known roles in conserving normal red cell shape and deformability were selected based on published reports of their association with hemolytic anemia due to red cell membrane defects including HE/HPP, with any mutations identified confirmed through Sanger sequencing [14, 17–23]. These genes, and their linked membrane proteins with disease-associations are listed in Table 1.

DNA samples of the nine patients were fragmented by Covaris technology and the whole exome sequencing was performed on BGISEQ platform. Raw data was stored in FASTQ format [24], low quality reads were removed, and high-quality reads (Q20 and Q30) were obtained.

Burrows-Wheeler Aligner (BWA) was used to align the clean reads per sample to the human reference genome (for ex: NCBI 1000 Genomes Project, dbSNP, ENCODE (The Encyclopedia of DNA Elements)). Picard tools were used to remove the duplicate reads. The sequencing depth and coverage for each individual were calculated based on the alignments (SAM files) [24].

The SAM files were processed using GATK and Picard tools. SNPs (single nucleotide variants/polymorphisms) and InDels (insertions and deletions) were simultaneously called via local de novo assembly of haplotypes using the HaplotypeCaller of GATK (v3.6). After quality score recalibration and removal of low-confidence variants, all SNPs and INDELs were annotated using SnpEff tool [24–26]. Detected variants were annotated and prioritized according to their presumed relevance to disease from publicly available databases and/or reports in published literature (gene-based annotation). Additionally, the effect of a variant was estimated in silico using mutation prediction software (SIFT (Sorting Intolerant From Tolerant), Polyphen, MA (Mutation Assessor), LRT (Likelihood Ratio Test), MT (Mutation Taster), and FATHMM (Functional Analysis Through Hidden Markov Models)) [26]. All the variants identified with the sequencing platform were classified using ACMG/AMP (American College of Medical Genetics and Genomics and the Association for Molecular Pathology) guidelines [27]. Figure 1 outlines the steps of the whole exome sequencing analysis procedure from generating the raw reads to variant calling.

Results

The study group comprised 5 males and 4 females with ages ranging from 2 to 46 years including 5 pediatric patients and their laboratory data are shown in Table 2. Figure 2 shows the morphology of red cells in one index case with high numbers of elliptocytes in a peripheral blood film.

On average, 99.9% of the sequenced DNA was mapped successfully. 58.97 Mb target regions were captured for variant calling. The average GC content was 47.36%. The predicted significant single nucleotide polymorphisms (SNPs) and the indels in the target genes of the nine selected patients are displayed in Table 3 and Table 4 respectively.

Discussion (case summaries)

Analysis of laboratory findings revealed that patients generally presented with mild/moderate anemia and prominent alterations of red cell indices. These abnormalities included increased red cell count and red cell distribution width (RDW) with reduced mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Among the indirect markers of hemolytic anemia (reticulocytes, bilirubin, and LDH), only reticulocytes were consistently increased (except in one patient). Serum LDH and bilirubin are less sensitive markers and were elevated in a relatively small subset of cases. Only two patients showed significant alteration of all parameters. Interestingly, patient #9 had a normal hemoglobin level but showed a markedly elevated RBC count

Table 1 Genes selected for next generation sequencing

Gene	Chromosome location	No. of exons	Membrane protein	Disease association
ANK1[17]	8p11.21	42	Ankyrin	Hereditary spherocytosis
SPTB[18]	14q23.3	38	β-spectrin	Spherocytosis type 2, hereditary elliptocytosis, and neonatal hemolytic anemia
SPTA1[19]	1q23.1	52	α-spectrin	Elliptocytosis-2, pyropoikilocytosis, and spherocytosis type 3
EPB41[20]	1p35.3	22	Protein 4.1	Elliptocytosis-1
ABCG5[21]	2p21	15	Sterolin-1	Sitosterolemia, stomatocytosis
ABCG8[21]	2p21	13	Sterolin-2	Sitosterolemia, stomatocytosis
RHAG[22]	6p12.3	11	Rh-associated glycoprotein	Rh-null hemolytic anemia, regulator type; stomatocytosis
PIEZO1[23]	16q24.3	51	Mechanosensitive ion channel protein	Dehydrated hereditary stomatocytosis

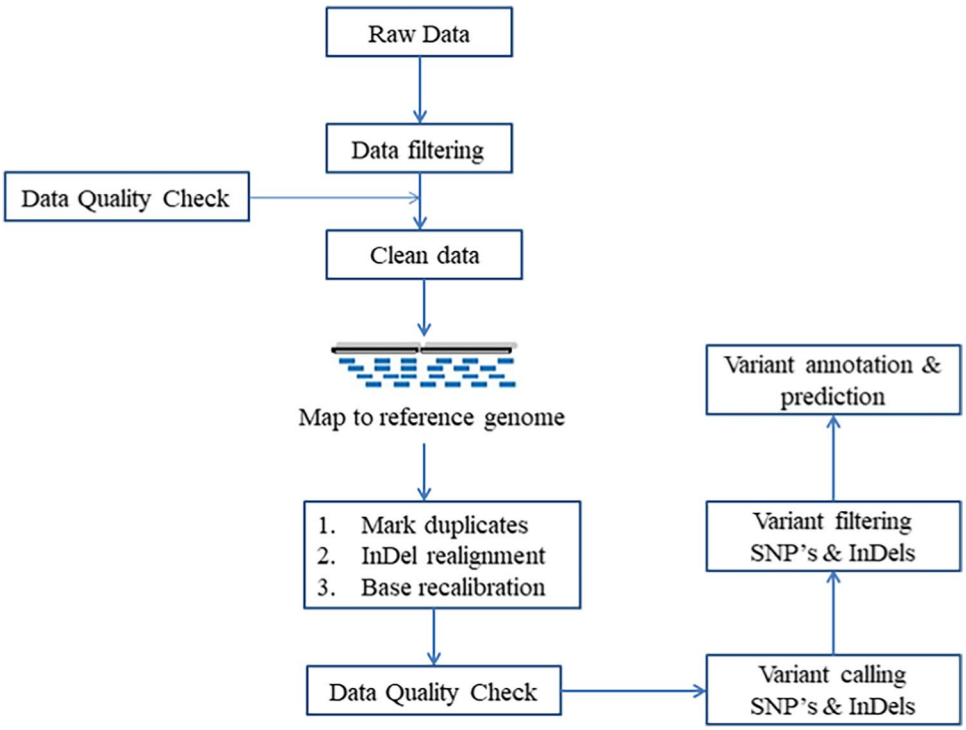

Fig. 1 Pipeline of whole exome sequencing analysis (from raw reads to variant calling)

with significantly altered hematologic and biochemical parameters. Taken together, these results suggest that a state of compensated hemolysis producing mild/moderate anemia characterizes most of our patients, a situation similar to published observations from other geographic regions [28].

Patient #1 was moderately anemic (hemoglobin 9.1 g/dL). DNA sequencing revealed a mutation of unknown significance (ClinVar database) in the carboxyl-terminal EF domain of SPTA1 (p. Lys2368Asn). The function of this EF calmodulin-like domain is not yet known; however, sph1J/sph1J mice with mutated EF domain have been reported to have very fragile red cells, suggesting that the domain is critical for skeletal integrity of erythrocyte. Reports suggest that the EF domain of SPTA1 binds to protein 4.2 [29]. In this patient the substitution of a conserved positively charged lysine by a polar (non-charged) arginine is expected to interfere with the binding affinity of SPTA1 to protein 4.2. The α^{LELY}-associated mutations were also noted in this patient, providing further evidence of the molecular basis of the disorder.

Patient #2 also presented with moderate anemia and similar mutations in SPTA1 as in patient #1 (c.7104G > T and α^{LELY}). Additionally, a Gly567Asp mutation in the hydrophilic N terminal of EPB41 was also noted in this patient. So far this SNP in EPB41 has not been reported in public databases; however, the mutation prediction software predicts it to be a potential VUS, probably because of the role of N-terminal of the protein 4.1 in interacting with spectrin and actin [30]. A homogenous mutation p. His1374Arg in SPTB was also seen in this patient. ClinVar database records this mutation as benign/likely benign based on it being reported in one HS and one HE patient (Illumina sequencing). An indel (disruptive inframe deletion) in the PIEZO gene (p.Glu756del/c.2268_2270delGGA) was also seen in patient #2. Deletion of this codon reportedly leads to partial gain-of-function phenotype of the PIEZO mechanosensitive ion channel even in the heterozygous condition [31, 32]. It is common in individuals of African origin and is reported to cause hereditary xerocytosis, characterized by RBC dehydration and anemia [33]. Ilboudo et al. (2018) reported the association of this mutation with increased RBC density due to dehydration and hemolysis in sickle cell disease [32]. This variant was also reported in a series of patients by NGS studies but evidence of functional abnormality is lacking [34]. This mutation has not yet been reported in association with HE/HPP. Moderate anemia that was seen in patient #2 could be related to this mutation in PIEZO gene with the compound effect of several associated heterozygous mutations observed in this patient. However, Rooks et al. (2019) did not observe any erythrocytic phenotype alteration in sickle cell disease patients with the PIEZO1 E756del polymorphism [35].

Patient #3 exhibited a homozygous deleterious variant of SPTA1 wherein amino acid leucine is substituted with proline and possibly this explains anemia with significant evidence of hemolysis (high LDH, reticulocytes, and bilirubin) in this patient. The L260P variant in the SPTA1 gene has been reported in a patient with elliptocytosis [36]. This amino acid substitution occurs at a position that is conserved across species. Functional studies indicate that this

Table 2 Demographic and hematological data of the study subjects*

Patient #	Gender	Age (yrs)	RBC 3.9–5.2 ($\times 10^{12}$/L)	Hb 12–16.5 (g/dL)	MCV 80–97 (fL)	MCH 27–33 (pg)	MCHC 30–37 (g/dl)	RDW 11.6–13.7 (fL)	Reticulocytes		Bilirubin 5–20 (mmol/L)	LDH 135–214 (IU/L)
									0.5–1.5 (%)	30–82 ($\times 10^9$/L)		
1	F	3	5.55	9.1	65.9	16.4	24.8	22.1	2.2	123	7	250
2	F	2	5.25	9.7	66.8	18.6	27.8	22.5	3.8	202	9	125
3	M	8	4.31	10.1	73.7	32.4	31.7	27.3	8.2	352	66	496
4	M	46	6.66	11.4	65.6	17.2	26.2	18.4	7.2	311	8	115
5	F	31	6.10	11	63.3	18.1	28.5	20	3.6	221	6	153
6	F	34	5.49	11.2	71.1	20.4	28.6	21.9	2.5	139	3	152
7	M	7	6.12	11.1	60.9	18.2	29.8	14.4	1.8	106	7	226
8	M	13	6.17	12	67.6	19.5	28.9	15.7	0.5	31	10	142
9	M	33	7.13	15	65.7	19.7	30	28.3	3.2	240	78	322

*Values related to laboratory parameters in the column headers relate to reference ranges

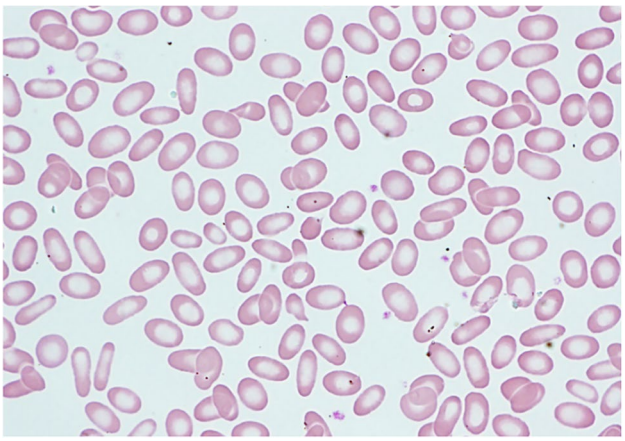

Fig. 2 Elliptocytosis: typical peripheral blood smear of an index case (Wright stain, × 1000)

variant form of spectrin shows impaired self-association to form oligomers [37]. A heterozygous PIEZO1 mutation p.Arg2476Cys was also seen in patient #3. The arginine at codon 2476 is moderately conserved and computational analyses (SIFT, PolyPhen-2) predict that this variant is deleterious. However, based on the available information, the clinical significance of this variant is uncertain [37]. This mutation has not been reported in association with HE/HPP.

Patient #4 had α^{LELY} associated with a heterozygous, reportedly benign, mutation in SPTA1 (p.Ser1163Ala). A heterozygous variant of uncertain significance with proline to leucine substitution in the trans membrane region of the ABCG8 occurred, was also noted. ABCG8 mutations are commonly reported in AR sitosterolemia and AD xanthelasma. Notably, single heterozygous ABCG8 variation in addition to α^{LELY} may not be sufficient to explain extreme hemolysis as seen in this patient [38]. However, possibility that association of this heterozygous mutation in SPTA1 (p.Ser1163Ala) becomes deleterious when acting in trans with the α^{LELY} allele as described by Agarwal et al. cannot be ruled out [11].

Patients #5 and #6, both adult females in reproductive age-groups, exhibited mild anemia with a known deleterious variant form of SPTA1 in heterozygous condition with a substitution of leucine with proline in the N-terminal domain. The significance of L260P variant of SPTA1 has been discussed earlier [36, 37]. This heterozygous SPTA1 mutation was associated with the α^{LELY} abnormality in both patients and this likely explains mild anemia with reticulocytosis in these patients [15]. Patient #5 also had a SPTB (p.Asn1151Asp) mutation, a likely benign variant, in homozygous condition.

For *patient #7*, mutation analysis of the sequence data revealed benign SNP mutations in SPTB (p. Asn1151Asp in homozygous, p. Gly1408Arg in heterozygous condition). A SNP in ANK1 was also seen. The mutation p.

Table 3 Candidate variants (SNPs) for each patient

Patient #	Gender, age (yr)	Hb g/dL	Gene	Nucleotide	Protein	Zygosity	Interpretation	Protein damage prediction by in silico tools*
1	F, 3	9.1	SPTA1	c.7104G > T	p.Lys2368Asn	Hetero	Likely benign (variant of unknown significance)	Polyphen B, MA M, LRT N, MT D
			SPTA1	c.5572C > G	p.Leu1858Val	Hetero	α^{LELY}	SIFT D, Polyphen B, MA M, LRT N, MT P, FATHMM T
			SPTA1	c.6531-12C > T	Intron variant	Hetero		
2	F, 2	9. 7	SPTA1	c.7104G > T	p.Lys2368Asn	Hetero	Likely benign (variant of unknown significance)	Polyphen B, MA M, LRT N, MT D
			SPTA1	c.5572C > G	p.Leu1858Val	Hetero	α^{LELY}	SIFT D, Polyphen B, MA M, LRT N, MT P, FATHMM T
			SPTA1	c.6531-12C > T	Intron variant	Hetero		
			EPB41	c.1700G > A	p.Gly567Asp	Hetero	Novel, Probably a variant of unknown significance	SIFT D, Polyphen B, MA L, LRT N, MT D, FATHMM D
3	M, 8	10.1	SPTA1	c.779 T > C	p.Leu260Pro	Homo	Deleterious	SIFT D, Polyphen D, MA H, LRT N, MT A, FATHMM T
			PIEZO1	c.7426C > T	p.Arg2476Cys	Hetero	Deleterious	SIFT D, Polyphen D, MA M, LRT D, MT D, FATHMM T
			SPTB	c.3451A > G	p.Asn1151Asp	Homo	Benign/likely benign	SIFT T,,Polyphen B, MA N, LRT N, MT P, FATHMM T
4	M, 46	11.4	SPTA1	c.5572C > G	p.Leu1858Val	Hetero	α^{LELY}	SIFT D, Polyphen B, MA M, LRT N, MT P, FATHMM T
			SPTA1	c.6531-12C > T	Intron variant	Hetero		
			SPTA1	c.3487 T > G	p.Ser1163Ala	Hetero	Benign	SIFT T, Polyphen B, MA N, LRT N, MT B, FATHMM T
			ABCG8	c.1568C > T	p.Pro523Leu	Hetero	Uncertain significance	SIFT D, Polyphen B, MA M, LRT N, MT D, FATHMM D
5	F, 31	11	SPTA1	c.779 T > C	p.Leu260Pro	Hetero	Deleterious	SIFT D, Polyphen D, MA H, LRT N, MT A, FATHMM T
			SPTA1	c.5572C > G	p.Leu1858Val	Hetero	α^{LELY}	SIFT D, Polyphen B, MA M, LRT N, MT P, FATHMM T
			SPTA1	c.6531-12C > T	Intron variant	Hetero		
			SPTB	c.3451A > G	p.Asn1151Asp	Homo	Benign/likely benign	SIFT T,,Polyphen B, MA N, LRT N, MT P, FATHMM T
6	F, 34	11.2	SPTA1	c.779 T > C	p.Leu260Pro	Hetero	Deleterious	SIFT D, Polyphen D, MA H, LRT N, MT A, FATHMM T
			SPTA1	c.5572C > G	p.Leu1858Val	Hetero	α^{LELY}	SIFT D, Polyphen B, MA M, LRT N, MT P, FATHMM T
			SPTA1	c.6531-12C > T	Intron variant	Hetero		
			SPTA1	c.7104G > T	p.Lys2368Asn	Hetero	Likely benign (variant of unknown significance)	Polyphen B, MA M, LRT N, MT D
			SPTB	c.3451A > G	p.Asn1151Asp	Homo	Benign/likely benign	SIFT T,,Polyphen B, MA N, LRT N, MT P, FATHMM T

Table 3 (continued)

Patient #	Gender, age (yr)	Hb g/dL	Gene	Nucleotide	Protein	Zygosity	Interpretation	Protein damage prediction by in silico tools*
7	M, 7	11.1	ANK1	c.753C > A	p.Asn > Lys	Hetero	Conflicting interpretations of pathogenicity: Benign/likely benign/ uncertain significance	SIFT D, Polyphen D, MA N, LRT D, MT D, FATHMM T
			SPTB	c.3451A > G	p.Asn1151Asp	Homo	Benign/likely benign	SIFT T,,Polyphen B, MA N, LRT N, MT P, FATHMM T
8	M, 13	12	InDel					
9	M, 33	15	SPTA1	c.779 T > C	p.Leu260Pro	Homo	Deleterious	SIFT D, Polyphen D, MA H, LRT N, MT A, FATHMM T
			SPTB	c.3451A > G	p.Asn1151Asp	Homo	Benign/likely benign	SIFT T,,Polyphen B, MA N, LRT N, MT P, FATHMM T

F female, *M* male, *Hb* hemoglobin

*In silico tools and predictions: SIFT (Sorting Intolerant From Tolerant): D, damaging; T, Tolerated; Polyphen: D, probably damaging; P, possibly damaging; B, benign; N, neutral; MA (Mutation Assessor): H, high; M, medium; L, low; N, neutral; LRT (Likelihood Ratio Test): D, deleterious; N, neutral; U, unknown; MT (Mutation Taster): A, disease causing automatic; D, disease causing; N, polymorphism; P, polymorphism automatic; FATHMM (Functional Analysis Through Hidden Markov Models): D, damaging; T, tolerated

Table 4 Candidate variants (InDels) in the study subjects

Patient #	Gender, age (yr)	Hb g/dL	Gene	Indel	Interpretation	Homo/hetero	Reported in public databases
2	F, 2	9.7	PEIZO	p.Glu756del/ c.2268_2270delGGA	Disruptive inframe deletion	Hetero	Yes
8	M, 13	12	PEIZO	p.Glu756del/ c.2268_2270delGGA	Disruptive inframe deletion	Hetero	Yes

Asn251Lys/c.753C > A is classified as "likely benign or variant of uncertain significance" in the ClinVar database and needs to be investigated further. This –NH2 terminal membrane-binding domain of ankyrin-1 is composed of 24 tandem repeats of approximately 33 amino acids folded into a nearly spherical structure. The mutation from a polar amino acid Asn to a positively charged Lys is likely to alter its interaction with the phospholipid membrane, as described in a case with mutation in the N-terminal of insulin receptor reported by Kadowaki et al. wherein Asn to Lys substitution at position 15 of N-terminal (α-subunit) of insulin receptor retarded the post-translational processing of the receptor and impaired transport of the receptor to the plasma membrane, thereby reducing the number of receptors on the cell surface [39]. So far there are three records of this ANK1 variant reported in cases of spherocytosis (benign/VUS) [38].

Patient #8 had normal hemoglobin and demonstrated a deletion in the PIEZO gene (p.Glu756del/c.2268_2270delGGA) leading to partial gain-of-function phenotype of PIEZO, identical to that in patient 2 [32–34].

Mutation analysis of sequence data of *patient #9* revealed a homogenous deleterious variant form of SPTA1 with substitution of leucine with proline in the N-terminal domain (L260P). L260P variant of SPTA1 was shown to impair self-association to form oligomers [36, 37]. Likely benign SPTB variants (p.Ser439Asn, p.Asn1151Asp) were also seen in homozygous condition. As the patient had normal hemoglobin in spite of presence of a homogenous deleterious variant form of SPTA1 with significant indicators of hemolysis (elevated reticulocytes, LDH, and bilirubin). This patient probably had a well-compensated hemolysis, as seen in many asymptomatic cases of HE/HPP [15].

Conclusion

Traditional sequencing studies have previously reported on the commonly mutated genes observed in HE and HPP (SPTA1, SPTB, and EPB41) [11, 37, 40, 41]. In the present

study we employed NGS to investigate 9 patients who were characterized by analysis of the whole exome sequencing data of 8 target genes linked to erythrocyte membrane structural proteins which reports on other plausible variants (ANK1, ABCG8, and PEIZO) to be associated with this condition.

As expected, in Bahraini HE/HPP patients, SPTA1 was also the most frequently mutated gene. The most commonly associated molecular signatures of HE in this small group of patients were as follows: (1) the deleterious L260P variant of SPTA1 seen in 4/9 patients with one patient (#3) having this mutation in homozygous form; (2) the α^{LELY} allele that was associated with compound heterozygous mutations in SPTA1 in five patients. This included a variant of uncertain significance in SPTA1 (p. Lys2368Asn / c.7104G > T) that was seen in heterozygous state in three patients (#1, #2, and #6).

SPTB variants were reported in seven patients. A likely benign variant of SPTB (p. Asn1151Asp/ c.3451A > G,) in homozygous state was seen in five patients (#3, #5, #6, #7, and #9), in addition to other SNPs in SPTB. Although predicted to be *likely benign*, the possibility of these being deleterious mutations cannot be excluded completely. The prevalence of these variants of SPTA1 and SPTB in HE/HPP patients in this region needs to be further explored.

A potential variant of EPB41 that could have deleterious impact on the function of the protein was also predicted with in silico tools in one patient (#2). Additionally, a novel variant of EPB41 (p. Gly567Asp /c.1700G > A), predicted to have potential deleterious impact, was also noted in patient #2. Patient #7 had a VUS in the ANK1 gene p. Asn251Lys/c.753C > A, pathogenicity of which has been reported as conflicting. Patient #4 had a VUS in ABCG8 gene (p. Pro523Leu/ c.1568C > T). A heterozygous deleterious PIEZO1 mutation (p. Arg2476Cys/ c.7426C > T) was seen in patient #3; however, this mutation has not been reported in association with HE/HPP. Finally, an indel abnormality (disruptive inframe deletion) in PIEZO leading to RBC dehydration was reported in two patients (#2 and #8). The association of this PIEZO mutation with HE/HPP cases has not been described so far in the literature.

The limitations of the sequence analysis employed in this study are that many etiologic mutations may be located in noncoding regions, such as regulatory or deep intronic regions that cannot be detected by means of whole-exome sequencing.

Evidence of phenotypic heterogeneity is seen even in this small sample size: two patients had normal-for-age hemoglobin whereas others were mildly anemic. All showed elevated reticulocyte count suggestive of active marrow compensation, but other hemolytic parameters (LDH, bilirubin) were variably abnormal. The markedly heterogenous and complex molecular abnormalities associated with membrane proteins in this condition may well explain the phenotypic and clinical variability — but is far from elucidation.

Author contribution Durjoy K. Shome: Conceptualization, supervision, writing — review and editing.
Priya Das: Data curation; formal analysis, writing — original draft.
Ghadir A. Akbar: Funding acquisition; investigation; methodology.
Safa Taha: Investigation; methodology supervision.
Ameera Radhi: Case screening and morphologic examination.
Khulood Al-Saad: Clinical investigations and data.
Rehab Helmy: Case screening and morphologic examination.

Data availability All the data will be available upon request.

Declarations

Ethics approval The study was approved by the Research and Research Ethics committees of the College of Medicine and Medical Sciences of Arabian Gulf University, Ministry of Health, SMC and KHUH prior to obtaining the samples from the patients.

Competing interests The authors declare no competing interests.

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