SPECIAL REPORT

Solving a cold case of haemolysis: Back to the basics

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ABSTRACT

Membrane disorders comprise an important group of inherited haemolytic anaemias. Diagnostic work-up starts with examination of the blood smear, followed by osmotic gradient ektacytometry. In special cases DNA analysis is performed to confirm the diagnosis. For this purpose a next-generation sequencing-based method has been developed. The combination of these techniques established the correct diagnosis in a case of haemolytic anaemia of unknown cause.

KEYWORDS

Hemolytic anemia, blood smear, next generation sequencing

What is known on this topic?
Haemolytic anaemias due to abnormalities of the erythrocyte membrane comprise an important group of inherited disorders. The 'gold' standard for the detection of various disorders of the red cell membrane is osmotic gradient ektacytometry.

What does this add?
Next-generation sequencing can be used in the diagnostic work-up of haemolytic anaemia after the blood smear and ektacytometry. This is the first report of a case of spherocytosis / stomatocytosis solved by next-generation sequencing.

INTRODUCTION

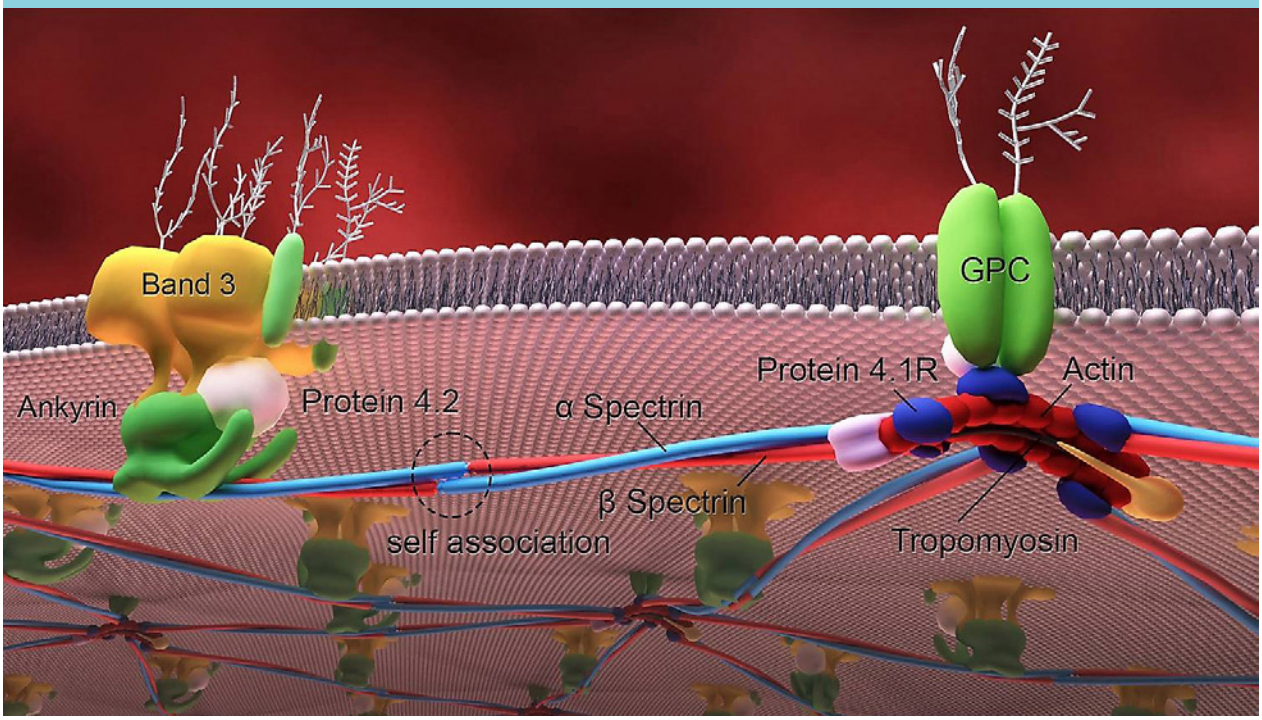
Many skills in jobs, art forms and sports are based on the application, repetition and training of elementary principles. The following case report reminds us to pursue the basic steps in elucidating the cause in a patient with haemolytic anaemia.

Erythrocyte membrane abnormalities are a well-known cause of hereditary haemolytic anaemia. The specific make-up of the red cell membrane accounts for its remarkable deformability and enables the cell to pass through small capillaries with a diameter of only one-third of its own. The membrane consists of a lipid bilayer in which transmembrane proteins are located which anchor the bilayer to an intracellular cytoskeleton. The latter forms a two-dimensional hexagonal filamentous meshwork, mainly consisting of spectrin tetramers (*figure 1*). Spectrin tetramers consist of alpha- and beta-spectrin protein, linked in a head-to-head arrangement, and encoded by the *SPTA1* and *SPTB* genes. Another prominent membrane protein is band 3, encoded by the *SLC4A1* gene. Band 3 is located in the erythrocyte plasma membrane and is part of a multiprotein complex that acts as an attachment site for the cytoskeleton. Abnormalities of each component of the cytoskeleton and anchoring proteins compromise their interactions, often leading to characteristic shape changes.[1]

CASE PRESENTATION

A 20-year-old Caucasian woman presented with jaundice. Her previous history included an episode of haemolytic anaemia three years earlier during a parvo B19 infection. At that time laboratory investigation revealed no haemoglobinopathy and no deficiencies of glucose-6-phosphate dehydrogenase or pyruvate kinase. Erythrocyte membrane protein analysis revealed 'normal'

Figure 1. *Artist impression of the erythrocyte cytoskeleton*



signals for spectrin (RIA) and Band 3 (EMA/FACS). One year later a second haemolytic episode occurred during an Epstein-Barr virus infection.

Five days before consultation she became acutely ill with nausea, vomiting and diarrhoea when travelling in Spain. Symptoms abated spontaneously, but she became jaundiced. On examination she was not acutely ill, but icteric. Results of laboratory investigations pointed to haemolysis as the cause of the jaundice (*table 1*).

A presumptive diagnosis of mild infectious enterocolitis with episodic nonimmune haemolysis was made. A new systematic review was performed. The persistently decreased level of haptoglobin, increased bilirubin, and reticulocytosis were indicative of mild chronic haemolysis. The blood smear disclosed abnormal erythrocyte morphology with prominent presence of spherocytes and stomatocytes (*figure 2*). Osmotic gradient ektacytometry showed decreased EI_{max} and increased O_{min}, indicative for spherocytosis (*figure 3*).

Based on these results a novel next-generation sequencing (NGS) technique was applied to detect mutations in the seven genes most commonly associated with erythrocyte membrane disorders (*SPTA1, SPTB, SLC4A1, ANK1, EPB41, EPB42,* and *RHAG*). This analysis revealed compound heterozygosity for two pathogenic *SPTA1* gene mutations: c.2806-13T>G (α-spectrin$^{St\ Claude}$) and c.4339-99C>T (α-spectrin^{LEPRA}). These mutations have previously been associated with autosomal recessively inherited poikilocytic anaemia and spherocytosis, respectively. In addition, the patient was found to be heterozygous for a c.118G>A, p.(Glu40Lys) missense mutation in *SLC4A1* which is associated with autosomal recessively inherited spherocytosis. The two α-spectrin mutations were present on separate alleles (*in trans*) as confirmed by DNA analysis of the parents.

Figure 2. *A May Grünwald Giemsa stained bloodsmear made after recovery of the patient showing abundant spherocytes (arrows) and stomatocytes (arrowheads)*

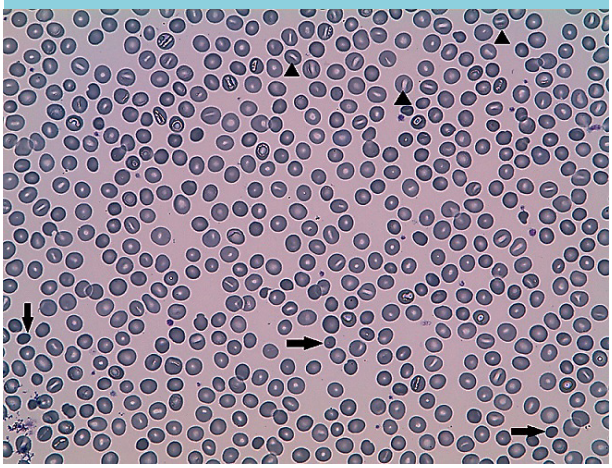
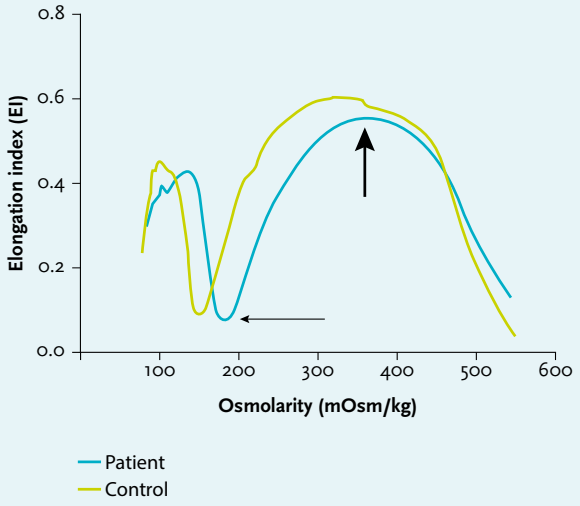


Table 1. *Laboratory results*

Haematology	Value	Reference range
ESR	15	<20 mm/h
Haemoglobin	7.1	7.5-10 mmol/l
Haematocritt	0.33	0.36-0.46 l/l
MCV	99	80-100 fl
RDW	66	39-52.3 fl
MCH	2.1	1.6-2.1 fmol/l
CHC	21.3	19-23 mmol/l
Erythrocytes	3.38	4.0-5.2 /pl
Reticulocytes	157	25-110 /nl
Leucocytes	6.7	4.0-10.0 /nl
Basophils	0.02	<0/2 /nl
Eosinophils	0.06	<0/4 /nl
Neutrophils	4.33	1.5-7.5 /nl
Lymphocytes	1.88	1.0-3.5 /nl
Monocytes	0.52	0.1-10.0 /nl
Immature granulocytes	0.9	<0.5 /nl
Thrombocytes	205	150-400 /nl
APTT	26	26-34 seconds
Prothrombin time	13	12-15 seconds
Chemistry		
Creatinine	61	50-90 umol/l
Albumin	43.1	35-55 g/l
C-reactive protein	2.4	<10 mg/l
Total bilirubin	114	<17 umol/l
Direct bilirubin	46	<5 umol/l
Alkaline phosphatase	77	35-105 U/l
γGT	54	<40 U/l
ASAT	129	<30 U/l
ALAT	129	<35 U/l
LDH	570	<250 U/l)
Haptoglobin	<0.1	0.4-2.0 g/l
DAT	Negative	Negative

ESR = erythrocyte sedimentation rate; MCV = mean corpuscular volume; RDC = red blood cell distribution width; MCH = mean corpuscular haemoglobin; CHC = cellular haemoglobin concentration; APTT = activated partial thromboplastin time; γGT = gamma glutamyltransferase; ASAT = aspartate aminotransferase; ALAT = alanine aminotransferase, LDH = lactate dehydrogenase; DAT = direct antiglobulin test.

Figure 3. *Osmotic gradient-ektacytometry by Laser-assisted Optical Rotational Cell Analyzer (LORCA) shows decreased maximal Elongation Index (Imax, thick arrow) and increased Omin (indicative of increased osmotic fragility, thin arrow). The pattern is typical for spherocytosis*



DISCUSSION

Examination of the peripheral blood smear was the key in establishing the correct diagnosis in this patient, despite normal results for red cell membrane disorder markers in previous investigations. In addition, osmotic gradient ektacytometry was performed which is the 'gold' standard for the detection of various disorders of the red cell membrane. By this method erythrocytes are exposed to both shear stress (by centrifugal force) and gradually changing osmotic conditions. These induced changes in the shape of the red cells are detected and render characteristic deformability profiles for elliptocytosis, spherocytosis, and stomatocytosis.[2]

Heterogeneity in the clinical severity and erythrocyte morphology of the hereditary haemolytic anaemias due to defective membranes is the rule as different proteins are involved in conjunction with a variety of genetic causes: deletions, nonsense, missense, null and low expression mutations and splicing variants.

The *SLC4A1* c.1178G>A mutation was first reported by Rybicki *et al.* and the protein harbouring the consequent p.(Glu40Lys) substitution was named band 3^{Montefiore}.[3] Their propositus was homozygous for this mutation and had hereditary spherocytosis. The defective band 3 protein probably resulted in faulty interactions with protein 4.2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Relatives who were heterozygous for the mutation were clinically unaffected. Whether the heterozygous state for this mutation is clinically relevant for the current case is uncertain, but an additive effect on the presentation of clinical features cannot be excluded.

Most mutations in the *SPTA1* gene affect the self-association site of the α- and β-spectrin molecules, leading to elliptocytosis. However, mutation at other sites can lead to pyropoikilocytosis and spherocytosis.

The *SPTA1*$^{\text{St Claude}}$ allele was first reported by Fournier *et al.*[4] Their homozygous propositus was symptomatic whereas the heterozygous parents were clinically and biochemically unaffected.

The *SPTA1*$^{\text{LEPRA}}$ allele was reported by Wichterle *et al.*[5] Their propositus was compound heterozygous for *SPTA1*$^{\text{LEPRA}}$ and *SPTA1*$^{\text{PRAGUE}}$ causing severe spherocytic haemolytic anaemia. The parents, heterozygous carriers of either of these mutations, were asymptomatic. Intriguingly, apart from spherocytosis our patient showed a large number of stomatocytes on the blood smear. This unique appearance may be related to co-inheritance of the band 3$^{\text{Montefiore}}$ allele.

It is highly likely that the mutations in spectrin and band 3 are responsible for the spherocytosis / stomatocytosis and the corresponding subclinical haemolysis. In patients with such membrane alterations intercurrent infections can exacerbate the haemolysis and provoke haemolytic crises. At the age of 15 and 16 years she had clinical jaundice which was attributed to haemolytic anaemia as part of serologically proven parvo B19 and Epstein-Barr virus infections, respectively, as the clinicians were unaware of the presence of structural changes in the red cell membrane. The last icteric episode occurred during an acute, self-limiting infectious enterocolitis (traveller's diarrhoea). It is, however, remarkable that she had no history of haemolytic crisis earlier in life since she must have suffered from other infectious childhood diseases.

Molecular analysis of the genes encoding red blood cell membrane proteins has long been hampered due to the size and number of the genes involved. With the advent of NGS techniques it has now become feasible to analyse many genes in parallel with high quality. For erythrocyte membrane disorders, the DNA diagnostics section of the UMC Utrecht has implemented NGS-based sequencing of seven genes known to be causally involved: *SPTA1, SPTB, ANK1, SLC4A1, EPB 41, EPB42* and *RHAG*. This gene panel analysis was validated and accredited under the ISO15189 standard and yields a mutation detection sensitivity of > 95% in the seven genes analysed. As such, a leap forward has been made in the facilitation of molecular genetic diagnostic analysis of patients with suspected erythrocyte membrane disorders. The fact that spectrin and expression of Band 3 protein on the erythrocyte membrane surface were previously determined as normal shows that a normal amount does not imply a normal structure and function of the protein.

CONCLUSION

This case presentation illustrates that a 'simple' examination of the blood smear, a basic laboratory test, is still a good starting point from which more sophisticated tests can be requested. The fact that the 'automated' haemocytometry report mentions results of a (leukocyte) blood cell 'differentiation' does not imply that the morphology of the red cells has been investigated. Moreover, most current automated cell counters do not recognise red cell shape alterations. Even if there has already been an extensive academic workup this should not discourage other physicians from performing a new systematic analysis in a cold case. Interaction with clinical chemists and the progress in DNA technology comes to aid the clinicians and patients at the bedside.

DISCLOSURES

The authors declare no conflicts of interest. No funding or financial support was received.

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