SOUTH EAST ASIAN OVALOCYTOSIS ASSOCIATED WITH BAND 3 GENE DELETION IN MALAY NEONATES IN KELANTAN

By

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VI LIST OF ABBREVIATIONS

α	Alpha
β	Beta
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
DRTA	Distal renal tubular acidosis
EDTA	Ethylenediamine tetracetic acid
H^+	Hydrogen ions
HCO_3^-/Cl^-	Bicarbonate/Chloride
HE	Hereditary Elliptocytosis
HPP	Hereditary pyropoikilocytosis
HUSM	Hospital Universiti Sains Malaysia
K^+	Potassium
PBF	Peripheral blood film
PCR	Polymerase chain reaction
SAO	Southeast Asian Ovalocytosis

VII ABSTRAK

Ovalositosis Asia Tenggara (SAO) tersebar luas di kalangan kumpulan-kumpulan etnik di Malaysia, Papua New Guinea, Filipina, Indonesia dan Thailand. Individu-individu heterozigus yang terkesan daripadanya tidak menunjukkan simpto-simptom tertentu tetapi satu penemuan yang baru telah menunjukkan bahawa individu-individu yang berkaitan telah menunjukkan kesan-kesan hemolisis dan pertambahan kandungan bilirubin di dalam aliran darah. Punca di peringkat molekular adalah disebabkan oleh kewujudan deletan 27- pasangan bes pada gen band 3 di kromoson 17. Gen ini juga disebut gen anion-exchanger 1 (gen AE1) dan hasil proteinnya yang berfungsi sebagai pengangkut anion-anion membran, contohnya klorida dan bikarbonat. Exspresi protin band 3 juga terdapat pada eritrosit dan sel-sel alfa (α) – interkalasi yang terdapat di tubuh liku distal pada ginjal.

Tujuan kajian ini ialah untuk membuat anggaran kewujudan deletan band 3 di kalangan bayi-bayi yang baru lahir di Kelantan dan untuk mengkaji perkaitan di antara penemuan-penemuan hematologi dengan kewujudan deletan band 3.

Satu ratus enam puluh orang bayi-bayi yang baru lahir di kalangan orang-orang Melayu telah dinilai untuk mencari keadaan ovalositosis Asia Tenggara melalui pemeriksaan darah periferi dan tindak balas polimeres berantai (PCR) untuk deletan protein band 3. Seramai tujuh daripada seratus enam puluh orang bayi telah dikenalpasti sebagai turut mengalami SAO (4.5%) dengan memeriksa gambaran darah periferi. Teknik PCR telah menunjukkan natijah yang sama.

Indeks-indeks hematologi menunjukkan bayi-bayi SAO telah mengalami kekurangan haemoglobin (Hb), jumlah sel darah (RBC) merah dan hemotokrit (HCT) yang signifikan. Nilai Hb pada bayi-bayi SAO ialah 13.5 ± 1.0 g/dL berbanding dengan nilai Hb sebanyak 15.0 ± 1.5 g/dL pada bayi yang tiada SAO (nilai p=0.008). Jumlah Rbc pada bayi SAO ialah 3.73 ± 0.2 x 10^{12}/L berbanding dengan jumlah Rbc pada bayi yang tiada SAO ialah 4.33 ± 0.47 x 10^{12}/L (nilai p=0.001). Nilai Hct bagi bayi SAO ialah 39.0 ± 3.2% berbanding dengan nilai pada bayi yang tiada SAO ialah 44.57 ± 5.7% (nilai p=0.013). Bayi-bayi yang menjadi kes-kes SAO juga telah menunjukkan pertambahan yang signifikan didalam nilai purata sel hemoglobin (MCH) dan jangka distribusi sel darah merah (RDW). Bayi-bayi SAO telah menunjukkan nilai MCH sebanyak 36.1 ± 0.3 pg berbanding dengan bayi-bayi yang tiada SAO menunjukkan nilai sebanyak 34.9 ± 2.1pg (nilai p=0.000). Nilai RDW pada bayi-bayi SAO ialah 19.2 ± 1.1 berbanding dengan nilai RDW pada bayi-bayi yang tiada SAO ialah 17.3 ± 1.6 (nilai p=0.001).

VIII ABSTRACT

Southeast Asian Ovalocytosis (SAO) is widespread in certain ethnic groups of Malaysia, Papua New Guinea, Philippines, Indonesia and Thailand. Affected heterozygous individuals are asymptomatic but a recent paper indicates that in some cases, individuals present with signs of increasing haemolysis and hyperbilirubinaemia. The underlying molecular defect is due to the presence of a 27-bp deletion of the band 3 gene on chromosom 17. This gene is also described as anion-exchanger 1 gene (AE1 gene) and its protein product serves as a membrane transporter of anion, namely chlorides (Cl^-) and bicarbonates (HCO_3^-). Band 3 protein is also expressed in the alpha (α) – intercalated cells of the tubules in the kidney.

The aims of this study were to investigate the presence of band 3 deletion in Malay neonates in Kelantan and to investigate the haematological findings associated with the presence of band 3 gene deletion.

One hundred and sixty Malay neonates were evaluated in this study for SAO by peripheral blood film (PBF) examination and underwent polymerase chain reaction (PCR) for band 3 gene deletion. Seven out of one hundred and sixty cases were detected to have SAO (4.5%) by PBF and these seven cases were also detected to have band 3 gene deletion at the molecular level.

The haematological indices showed that the SAO affected babies had significantly reduced haemoglobin (Hb), total red blood cell counts (Rbc), and

haematocrit (Hct). The haemoglobin values were 13.5 ± 1.0 g/dL in SAO affected baby compared to 15.0 ± 1.5 g/dL in non SAO baby (p-value=0.008). Total Rbc count in SAO babies is 3.73 ± 0.2 x 10^{12}/L compared to 4.33 ± 0.47 x 10^{12}/L in non SAO baby (p-value=0.001). The Hct in SAO baby is 39.0 ± 3.2% compared to 44.5 ± 5.7% in non SAO baby (p-value=0.013). These SAO cases also had increased mean cell haemoglobin (MCH) and red cell distribution width (RDW). The SAO affected babies had MCH of 36.1 ± 0.3 pg as compared to the non SAO babies who had MCH of 34.9 ± 2.1 pg (p-values=0.000). The RDW in SAO babies had a values of 19.2 ± 1.1 when compared to a value of 17.3 ± 1.6 in non SAO babies (p-value=0.001).

1 INTRODUCTION

South east Asian Ovalocytosis (SAO) is an autosomal dominant inherited red cell disorder widespread in certain ethnic groups of Malaysia, Papua New Guinea, Philippines, Indonesia and Thailand.[1] Affected heterozygous individuals are asymptomatic but a recent paper indicates some affected individuals may present with signs of increasing hemolysis and hyperbilirubinemia.[1] The homozygous state is assumed to be lethal in utero.[2] Among the Malays in Ulu Jempul District in Kuala Pilah, West Malaysia, the frequency of SAO is high, about 13.2 %.[3]

SAO is a form of hereditary elliptocytosis in which the majority of red cells are oval in shape with a length to width ratio greater than 1 but less than 2, often associated with stomatocytes and knizocytes in a peripheral blood film.[4,5] The presence of *25%* ovalocytosis in which some are oval stomatocytes, oval macrocytes and cells containing longitudinal slit or transverse ridge in peripheral blood film is an accepted basis for the clinical or epidemiological diagnosis of SAO.[5] They are usually picked up as an incidental finding from a routine blood film examination.[6]

The underlying molecular defect in SAO is a deletion of 27 basepairs in the band 3 gene on Chromosome 17 resulting in the deletion of 9 amino acids (400-408) at the boundary between the cytoplasmic and the first transmembrane domains of band 3 protein This defect is tightly linked to band 3 Memphis polymorphism, a point mutation causing substitution AAG → GAG (lysine - glutamic) in codon 56.[8,9]

PCR amplification of genomic DNA that contains the site of the deletion is able to verify the affected SAO individuals. In order to further the understand the associated haematological and molecular aspects of SAO, one hundred and sixty samples of normal Malay newborns in HUSM and Hospital Kota Bharu were studied by using hematological and PCR analysis.

1.1 Literature Review.

1.1.1 Structure of the Red Blood Cell.

The mature red blood cells is easily recognized because of its unique morphology. At rest, the red blood cells takes the shape of a biconcave disc with a mean diameter of 8 μm, a thickness of 2 μm and a volume of 90 fl. It lacks a nucleus or mitochondria, and 33 % of its contents are made of a single protein, haemoglobin. Intracellular energy requirements are largely supplied by glucose metabolism, which is targeted at maintaining hemoglobin in a soluble, reduced state, providing appropriate amounts of 2,3-diphosphoglycerate (2,3-DPG) and generating ATP to support membrane function. Without nucleus or protein metabolic pathway, the cell has a limited lifespan of 100 to 120 days. However, the unique structure of the adult red blood cell is perfect for its function, providing maximum flexibility as the cell travels through the microvasculature (Fig 1.1).[10]

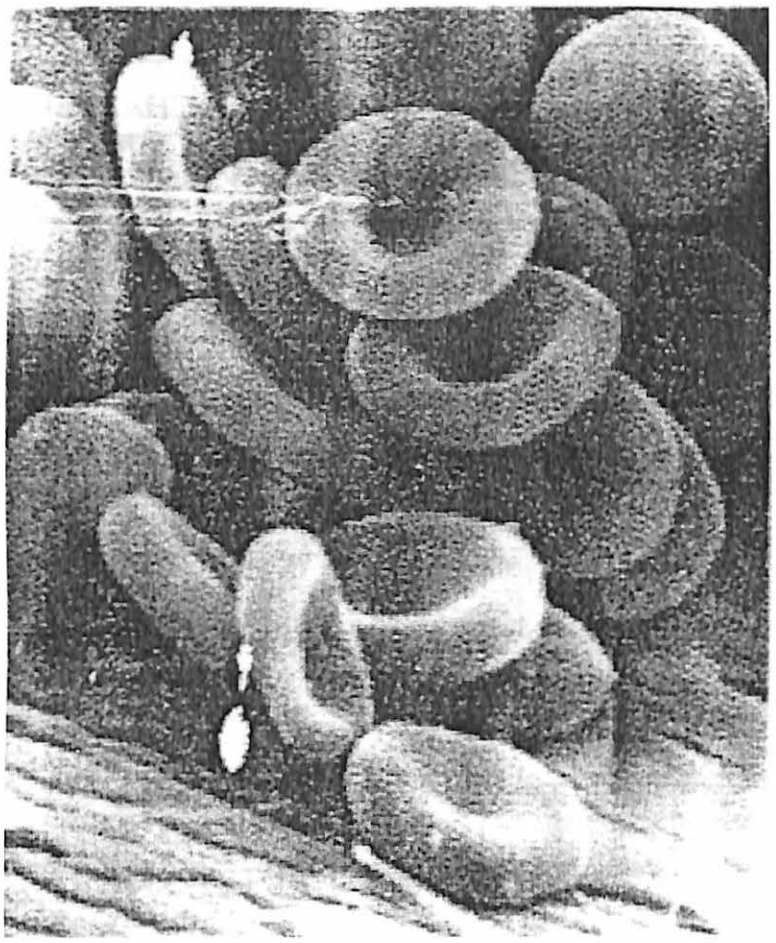

Figure 1.1 Red blood cell morphology.

[Adapted from James Morgan and Pamela Hanley (1998)]

1.1.2 Membrane

The shape, pliability and resiliency of the red blood cell is largely determined by its membrane. The structure of this membrane is illustrated in Fig 1.2. It is a lipid sheath, just two molecules thick, that consists of closely packed phospholipid molecules. The external surface of the membrane is rich in phosphatidylcholine, sphingomyelin and glycolipid, whereas the inner layer is largely phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol. This asymmetry is maintained by two transporters, ATP-dependent aminophospholipid translocase and floppase. Interference with these transporters results in a relocation of phosphatidylserine to the

cell surface with a resulting increase in the thrombogenic potential of the cell surface. Accumulation of excess phosphatidylserine on the red cell surface as a port of aging may also be responsible for macrophage destruction.

Approximately 50 percent of the red blood cell membrane is made up of cholesterol that is in equilibrium with the unesterified cholesterol in the plasma. Because of this, the cholesterol content of the membrane is influenced by plasma cholesterol levels, as well as by the activity of the enzyme lecithin cholesterol acyltransferase (LCAT), and bile acids. Liver disease patients with impaired LCAT activity accumulate excess cholesterol on the red blood cell morphology (targeting) and at times a shortened survival.[10]

The outer lipid membrane layer is affixed to a reticular protein network consisting of spectrin and actin. As shown in Fig 1.2, the integral proteins glycophorin C and band 3, which function as anion exchangers, extend vertically from the spectrin lattice work through the lipid layer to make contact with the cell surface. Spectrin heterodimers interact horizontally with protein 4.1 and complementary spectrin heterodimers to form a hexagonal lattice framework under the lipid bilayer. Defects in the vertical structure of the membrane (deficiency of spectrin, ankyrin, or band 3, or loss of lipid) result in spherocyte formation. Damage to the horizontal spectrin framework results in severe red cell fragmentation or mild elliptocytosis.[10]

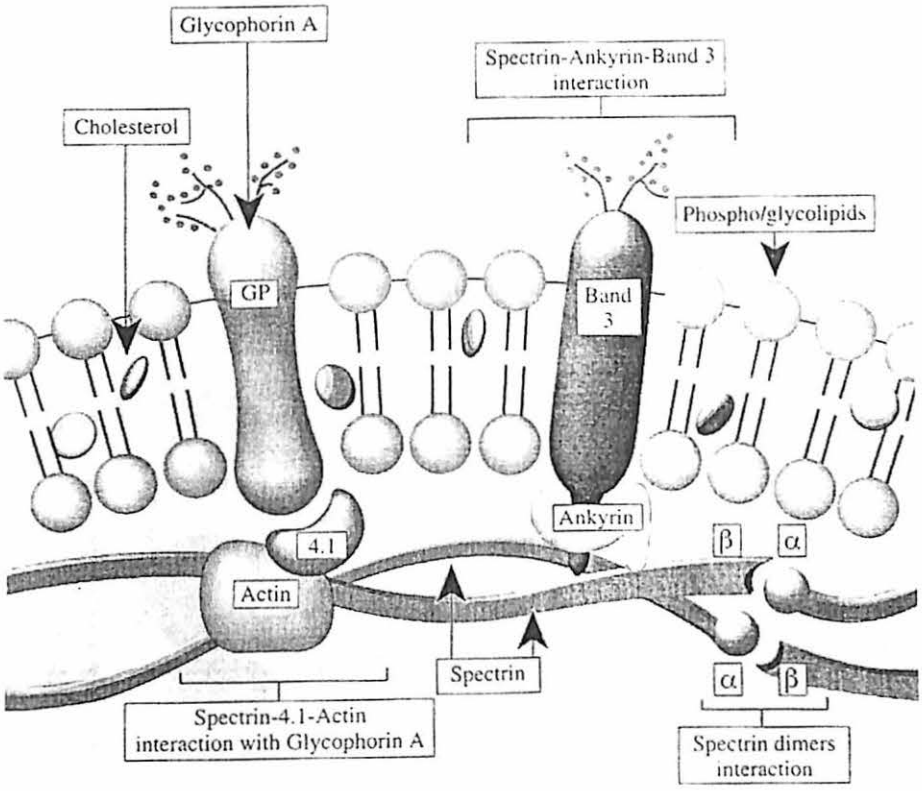

Figure 1.2 Red blood cell membrane structure.

[Adapted from James Morgan and Pamela Hanley (1988)].

The integral proteins and surface glycosphingolipids are also responsible for the cell's antigenic structure. More than 300 red blood cell antigens have now been classified with the ABO and Rh blood group antigens, being of primary importance in typing blood for transfusion. Antibodies against minor blood group antigens can result in increased red blood cell destruction by the reticuloendothelial cells.[10]

1.2 Hereditary Elliptocytosis.

A number of disorders that affect red cell shape may be grouped under the general title of hereditary elliptocytosis (HE). The characteristic feature is the more or less elliptical shape of the red cell, although this may vary from an almost spherical ellipse to grossly distorted cells. The phenotypes may be arranged in four groups, distinguished by their shape. They are common HE, hereditary pyropoikilocytosis (HPP), spherocytic HE and South East Asian Ovalocytosis.[11]

1.2.1 Common Hereditary Elliptocytosis

This is the most common type of membrane disorder that produces haemolysis. It has a frequency of about 1 in 2500 northern European Caucasians and 1 in 150 populations from some parts of Africa.[11]

1.2.2 Laboratory Findings

The typical morphology of the elliptocyte is shown in Fig. 1.3. The red cell survival is only slightly reduced and anaemia is rare. The reticulocyte count is not usually above 3%. Significant anaemia is seen in some patients, mainly those in whom there is co-inheritance of spectrin α^{LELY}. The osmotic fragility is normal or slightly reduced. There may be increased lysis in the 48-hour auto-haemolysis test, which is corrected by the addition of glucose.[11]

1.2.3 Clinical Features

Common (mild) HE is dominantly inherited. The elliptocytosis found in populations from parts of West Africa is thought to have a protective effect against falciparum malaria. Most patients are asymptomatic and the abnormality is found by chance. The spleen is occasionally palpable but not in most patients. A small proportion of patients with symptomatic anaemia may have to be considered for splenectomy.[11]

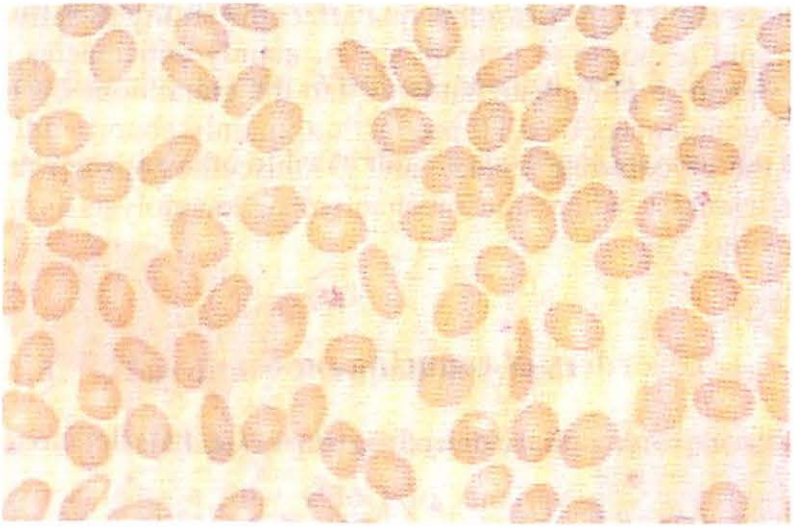

Figure 1.3 Common hereditary elliptocytosis

[Adapted from A. Victor Hoffbrond, S. Mitchell Lewis, Edward G.D. Tuddenham (1999)]

1.2.4 Pathogenesis

Many different gene mutations produce the phenotype of common HE. Usually, they affect the cytoskeleton network of interlinking tetramers through abnormalities of the self-association regions of spectrin caused by anomalies in the α or β-spectrin subunits or protein 4.1. These have been termed 'horizontal' interactions. A very mild type of HE is seen in red cells lacking glycophorin C and protein p55. The main effect

of these mutations is to increase the proportion of spectrin heterodimers to above the normal 5%. The degree of elliptical distortion and the rate of haemolysis correlate with the proportion of heterodimers. Spectrin extracts which have more than forty percent heterodimers are associated with severe haemolysis.

Spectrin α^{LELY} is a common polymorphism which alters α-spectrin mRNA splicing so that only about 50% of α-spectrin is produced. The polymorphism is harmless by itself, even in the homozygous state, since α-spectrin is normally made in considerable excess. When co-inherited with a defective HE, the effect is to greatly increase the ratio of heterodimers and hence increase the haemolysis.[11]

1.3 Hereditary Pyropoikilocytosis

Hereditary Pyropoikilocytosis is a rare phenotype of severely distorted and fragmented cells associated with moderate to very severe haemolysis (Fig. 1.4). It is inherited as a recessive disorder, which arises from inheritance of two abnormal α-spectrin genes either by homozygosity or compound heterozygosity. The condition may also arise from inheritance of a more severe structural spectrin defect with spectrin α^{LELY}. The haemolysis usually improves after splenectomy but some shortening of the red cell survival remains. Infants with some common HE disorders may show a blood picture of HPP in the neonatal period, which gradually reverts to the typical HE morphology.[11]

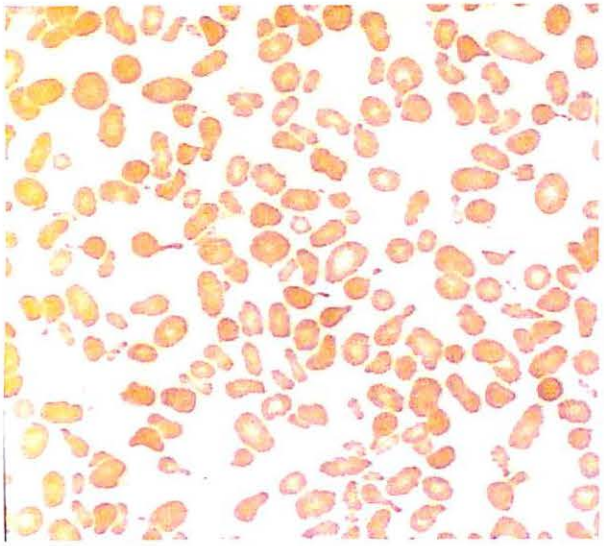

Figure 1.4 Hereditary pyropoikilocytosis.

[Adapted from A. Victor Hoffbrand, J.E. Pettit (2000)]

1.4 Spherocytic Hereditary Elliptocytosis

As the name implies, this phenotype is close to spherocytosis in appearance. There are none of the elongated or fragmented forms seen in common HE. The rounded elliptocytes are osmotically fragile (unlike common HE) and have a markedly shortened life; usually causing a mild to moderate anaemia. Splenectomy is curative. Defects which have been identified affect the β-spectrin subunit. Caucasian populations are affected.[11]

1.5 Stomatocytosis, Xerocytosis and Hydrocytosis

In some families with inherited haemolytic anaemia in whom the shape change of the red cell does not fit the above broad classification, the cause of the haemolysis is thought to lie in as yet unidentified anomalies of the membrane.

Patients with hereditary stomatocytosis have cup-shaped red cells, which, on dried films, appear to have a mouth like slit (stoma) instead of the round area of central pallor seen in normal red cells. Occasional stomatocytosis are seen in small proportion in a number of acquired haemolytic anaemias, but persistence of a stomatocytosis greater than 20% is pathognomonic of the inherited disorder. The characteristic feature of the red cells is a marked leakiness to both Na^+ and K^+, which leads to an increase in cell water. The term 'hydrocytosis' has been applied to these conditions. As with the other membrane disorders, there is marked clinical heterogeneity, which probably reflects the underlying genetic diversity. On the whole, patients have no or mild anaemia, although more marked haemolysis may be seen in some patients, possibly those who are homozygous. The blood film changes are particularly common in Australians of Greek origin.[11]

Patients whose red cells lack the Rh antigen (Rh_{null}) have a mild haemolytic anaemia with stomatocytosis. There is molecular heterogeneity in this syndrome also. The Rh_{null} phenotype may be due to homozygosity for a suppressor gene or to the presence of defects relating directly to the RhD genes themselves.

Other cases are described under the title of xerocytosis in patients in whom the red cells show some degree of shrinkage on the blood film and may have decreased osmotic fragility. The haemolyis is usually mild.[11]

1.6 South-East Asian Ovalocytosis

Since the first description of hereditary ovalocytosis in Malayan aborigines,[6] high frequencies of ovalocytosis have been reported in South East Asia.[4] Biophysical studies showed a strong correlation between increased membrane rigidity and decreased malarial parasite invasion in hereditary ovalocytosis in the Malayan aborigines.[12] Hereditary ovalocytosis is present in 5.1% of Malays.[13]

Southeast Asian ovalocytosis (SAO) is a dominantly inherited disorder that is widespread in certain ethnic groups of Malaysia, Indonesia, Papua New Guinea and the Philippines.[4] SAO red blood cells (RBCs) are ovalocytic in shape, rigid and resistant to invasion by different strains of malaria parasites.[18] It was suggested that the increased membrane rigidity of ovalocytes was most likely to be a consequence of a mutation in one of the principal components of the skeletal network, namely, spectrin, actin, ankyrin and protein 4.1.[9]

Affected heterozygous individuals are asymptomatic, with no clinically detectable haemolysis,[6] although one subject has been described with compensated haemolysis.[15]

The prevalence of SAO in other population groups is not known, but one Mauritian subject of Indian extraction and one African-American family have been reported.[15]

In some populations of Papua New Guinea and Malaysia, the frequency of SAO band 3 mutation is as high as 40%.[6] The absence of homozygosity for the SAO band 3

mutation in the subjects under this study raises a possibility that the homozygous state in incompatible with life.[2]

A high frequency of nonhemolytic hereditary ovalocytosis in Malayan aborigines is thought to result from reduced susceptibility of affected individuals to malaria. Indeed, Kidson et al, recently showed that ovalocytes from Melanesians in Papua New Guinea are resistant to infection in culture by the malarial parasite Plasmodium falciparum.[12]

Based on an epidemiologic study of Malayan aborigines from an area where malaria was endemic Baer et al (1976)[17] put forward the hypothesis that ovalocytosis which occurs with high frequency (~30%) in this population, might represent yet another red cell variant genetically selected by its associated protection against malaria. In order to define the mechanism of protection against parasite invasion in these ovalocytes, Mohandes N et al (1984) has been able to show that the resistance to invasion is directly related to the increased membrane rigidity of the ovalocytes and suggested that resistance to parasite invasion of ovalocytes in Malayan aborigines is the result of a genetic mutation that results in increased membrane rigidity.[12] Terence et al (1983)[18] also reported that erythrocytes from humans with Melanesian elliptocytosis are resistant to invasion by Plasmodium falciparum in vitro and epidemiological evidence suggests they may be resistant to P. vivax and P. malariae. Epidemiological data indicates that patients with elliptocytosis in Melanesia also have a lower parasitaemia and frequency of infection with other human malarias, namely P. vivax and P. malariae.[18]

Southeast Asian ovalocytosis status was determined in 1629 individuals originating form 12 different geographical areas of Papua New Guinea, representing different ethnic groups and degrees of malaria endemicity by using polymerase chain reaction amplification to demonstrate a 27 base pair deletion in the erythrocytes band 3 (AE 1) gene. The prevalence of erythrocyte band 3 gene deletion was determined to range from zero in both the lowland area of Wosera, East Sepik province and the highland region of Goroka, Eastern Highland's province to 35% on the north coast of Madang province. In general, the prevalence correlated well with altitude, being highest on the coast where malaria transmission is high, intermediate in the lowlands, and lowest in the non-malarious highlands. However, Worsera, a lowland area in the Sepik River plains, which is hyperendemic for malaria, was an exception in that no ovalocytosis was detected. These results largely confirm the prevalence rates that have been reported in the past using microscopy. In keeping with the autosomal dominant mode of inheritance, the male: female ratio was 1.02 and no homozygote was detected.

The condition is widespread in various ethnic groups in South-east Asia and extends to Papua New Guinea, where high prevalence has been described in the coastal and lowland areas. Traditionally, the diagnosis of this condition has been made by microscopical examination of peripheral blood films after Giemsa staining, a process that is both laborious and subjective. Using this method, the prevalence of hereditary ovalocytosis has been estimated to range from nearly zero in the highlands, where there is very little malaria transmission, to about 30% in the coastal area of Madang Province, where malaria is hyperendemic.[8,19] Similar surveys from the coastal areas of the Southern Papuan region report prevalence rates of 11% in the Port Moresby area, 22% in Pari, 17% in Hisiu (Amato, 1975), and 13% in the Gulf area.[4] However, examination

of peripheral blood films for the diagnosis of ovalocytosis has been associated with both inter- and intra-observer variation. Moreover, varying ovalocyte count cut-off points have been used in the past to define hereditary ovalocytosis. Although some investigations have taken an ovalocyte count of 50% or more to diagnostic, others have considered counts as low as 25% to sufficient for the diagnosis of ovalocytosis, while others have arbitrarily classified the condition into 3 different categories, namely, intermediate, ovalocytic and ovalocytic with haemolytic potential, thus implying heterogeneity. According to this classification, an ovalocyte count ranging between 10 and 25% is considered to be intermediate, 25 to 90% is considered ovalocytic and above 90% as ovalocytic with potential for haemolytic anaemia.[5] Alternatively, SAO has been simply classified into low frequency when the ovalocyte count is between 50 and 70%.[5] In the original descriptions of SAO in Malayan aborigines (Lie-Injo et al, 1985) and Papua New Guinea (Amato, 1975), an ovalocyte count of 25% was used as the cut-off point.

This inconsistency in diagnostic criteria, among other factors, may have in the past contributed to the controversy over the mode of inheritance of this red blood cell variant, with some suggesting autosomal recessive inheritance[4] and others an autosomal dominant mode.[5] Further, when low and high frequency ovalocytosis were distinguished, the low frequency ovalocytosis was considered to be inherited as an autosomal recessive trait and the high frequency ovalocytosis as an autosomal dominant. These problems have now been resolved by the delineation of the molecular pathology, which has been ascribed to a 27 base pair (bp) intraexonic deletion involving codons 400 to 408 of the erythrocyte band 3 (AE 1) gene, whereby heterozygosity leads to manifestation of ovalocytosis.[16] By using a polymerase chain reaction (PCR), it is

possible to amplify the deoxyribonucleic acid (DNA) fragment flanking the mutation and demonstrate its presence in affected individuals, thus providing a convenient, consistent and objective way of diagnosing the condition. Moreover, it has also confirmed the mode of inheritance to be autosomal dominant. In keeping with many other autosomal dominant conditions, no homozygous erythrocyte band 3 deleted individual has been ascribed to date, which suggests that homozygosity results in intrauterine death.[5]

1.7 Band 3

The membrane of the red blood cell has some 15 major protein constituents, one of them being band 3. This is a transmembrane protein, and gets its name from its relative position on gels after electrophoresis. Band 3 is central to the function of red cells, having two roles. It maintains the integrity and stability of the erythrocyte by anchoring the erythrocyte skeleton to the cell's lipid bilayer, and it increases the capacity of blood to carry CO_2, as HCO_3^-, from the tissues to the lungs – it does this because it is an anion exchanger, and indeed is also known as anion exchanger 1 (AE 1). People who are partially deficient in band 3 are not uncommon in the population, and they suffer from a form of haemolytic anaemia, known as hereditary spherocytosis, in which the shape of the red cells is distorted. But the complete absence of erythrocyte band 3 has never been described before and the protein has been regarded as being essential for life.

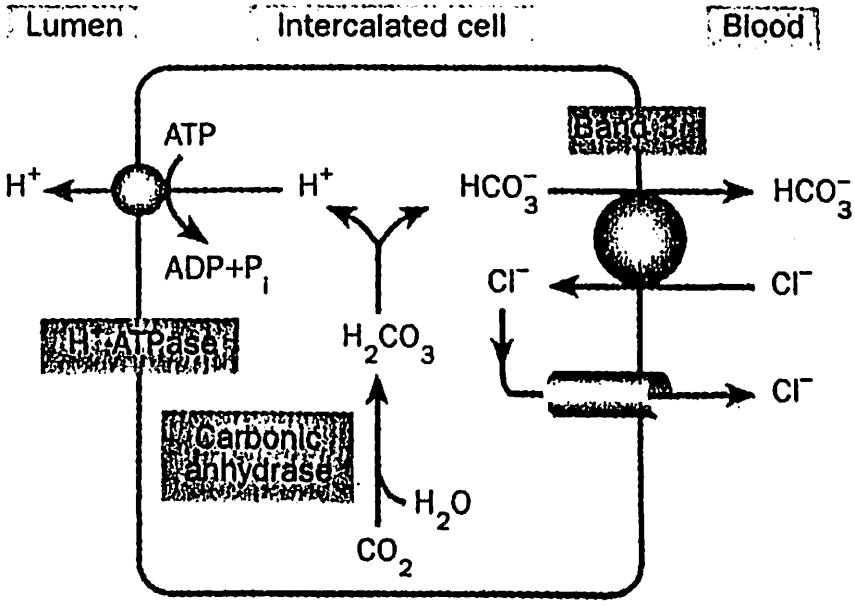

Figure 1.5 Function of band 3 in acid secretion by intercalated cells in the renal distal tubule.

The final portion of the kidney tubule – the distal tubule and collecting duct – secretes acid into the tubular fluid and also determines the final concentration of urine. This tubular epithelium has two main types of cell, the intercalated cells which secrete acid and the principal cells which regulate water permeability. The figure illustrates the mechanism of acid secretion by the intercalated cell. The apical H^+-pumping ATPase in the cell drives protons into the tubular lumen, where they are buffered by phosphate and ammonia in the luminal fluid. The depletion of intracellular protons (which would otherwise alkalinize the cell interior) is countered by a supply of protons derived from the intracellular hydration of $C0_2$ by carbonic anhydrase. This forms intracellular HCO_3^- which is transported into the blood in exchange for chloride by band 3, and the chloride ions that have moved into the cell leave through chloride-selective channels. (Adapted from Tanner M J A, Nature Vol 382, 1996, p 209)

Two reports now challenge this view – Inaba[24] and co-workers have found Japanese black cattle that are completely deficient in erythrocyte band 3, and a preliminary report by Peters et al describes the production by gene disruption methods, of mice that are likewise deficient. Some of these animals survive, although they have severe haemolytic anaemia and retarded growth.[23]

Band 3 is expressed only in red cells and acid-secreting cells in the kidney.[23] The protein contains two domains;[25] the amino-terminal portion binds the erythrocyte skeleton and the carboxy-terminal membrane domain carries out anion transport. The erythrocyte skeleton, a two-dimensional network of proteins located under the lipid bilayer, enables the cell to remain intact despite the physical stresses of the circulation.[26]

The band-3-deficient red cells showed very little anion transport. CO_2 is converted to HCO_3^- within erythrocytes by carbonic anhydrase. HCO_3^- is normally equilibrated between the cells and the blood plasma by the Cl^-/HCO_3^- exchange activity of band 3. However, in the band-3-deficient animals, HCO_3^- is restricted to the cells and this, together with the low haematocrit, allows only a small proportion of the blood volume to carry HCO_3^-. The resting adult band-3-deficient cattle (25% haematocrit) had mild acidosis – over-acidity with decreased blood HCO_3^-, increased blood CO_2 and blood pH reduced by 0.15 pH unit. The last may account for the retarded growth of the animals observed by Inaba et al. The acidosis especially severe in the newborn animals, because of their very low haematocrits, and may contribute to their high death rates.[23]

Band 3 is also involved in regulating whole-body acid-base balance by the kidney. Net nonvolatile acid clearance occurs by acidification of the urine, while net base clearance results in alkalinisation of the urine by secretion of HCO_3^-.[23] Acid secretion by the α-intercalated cells of the distal nephron requires Cl^-/ HCO_3^- exchange (see figure) and this process is mediated by band 3. Normally, calves can be acidify their urine to pH 5.3 – 5.6 in response to an acid load,[23] but despite their acidosis, the band-3-deficient cattle could not acidify their urine to less than pH 7.5.

This defective renal-acid secretion also impaires the animals' ability to correct acidosis. In normal animals, diet largely determines whether there is a net non-volatile acid or base excess . Carnivoures and Omnivoures with a high protein diet excrete an acidic urine; herbivores excrete and alkaline urine, because the organic anions in their leafy diet produce base as HCO_3^-. The normal controls for Inaba and colleagues' band-3-deficient cattle had urine pH 8.0 and were in base excess, and this dietary base excess may amelionate the acidosis in the cattle lacking band 3. in humans, renal tubular acidosis is treated by sodium bicarbonate.[23]

The band-3-deficient animals should be valuable for studying mammalian acid-base physiology. They might provide an animal model for human distal tubular acidosis – defective acid secretion in the distal tubule. This disease can originate in several ways and has a variety of clinical features, the most serious of which are kidney stones, bone disease and muscle weakness.[23]

Band 3 is the major integral protein on the red cell membrane that interacts with the membrane skeleton. Deficiency of band 3 is found in about 20% of American and

European patients with HS, but is more common in Japanese.[27] The disease is inherited as a dominant trait with relatively mild anaemia and spherocytosis. Many unsplenectomized patients also have a small population (0.2 – 2.3%) of mushroom-shaped or 'pincered' erythrocytes,[27,30] which are not seen in other forms of HS. A murine model of band 3 deficiency showed that band 3 is essential for stability of the membrane lipid bilayer but not for assembly of the membrane skeleton.[8]

Many changes in the band 3 gene that result in null mutations have been identified in patients with HS. Band 3 Hod ouin, Lyon, Noirtere, Osnabriick I and Trutnov have nonsense mutations that cause mRNA instability and band 3 deficiency.[27,32,33] Band 3 Bicētre II – Bohain, Briiggen, Fogia and Smichov have single nucleotide insertions in the coding region that produce a frameshift mutation, whereas band 3 Evry, Hobert, Napoli I, Princeton and Worcester have single nucleotide deletions.[34,35] Band 3 Campinas and Priban result from splicing defects and band 3 Prague occurs because of a 10-nucleotide duplication in the gene.[36,37]

Missense mutations or short in-frame deletions have also been found in HS patients. Many such mutations are located in the transmembrane domain and probably cause poor incorporation of band 3 into the membrane. In band 3 Bicētre I, Dresden, Hradec Kralore, Japlanec, Prague II and Prague III, substitution of highly conserved arginine residues positioned at the internal boundaries of transmembrane segments probably interferes with membranes with contranslational insertion of band 3 into the membranes of the endoplasmic recticulum.[8,35,38] In band 3 Benesor, Birmigham, Chur, Most, Napoli II, Okinawa and Philadelphia, other highly conserved amino acids crucial for stabilisation of band 3 within the lipid layer are substituted.[34] A 22-residue insertion

in the transmembrane domain in band 3 Milano prevents incorporation of the peptide into the membrane,[40] and a single amino acid deletion in the transmembrane domain is found in band 3 Osnabrück II.[8]

Missense mutations in the cytoplasmic domain of band 3 can interfere with its binding to other membrane skeleton proteins, resulting in a functional defect. In band 3 Nachad, a deletion of five amino acids from the ankyrin-binding site disrupts this binding.An amino acid substitution in the cytoplasmic domain in band 3 Fukuoka causes defective protein 4.2 binding. A compound heterozygote carrying both band 3 Fukuoka and band 3 Okinawa has complete absence of protein 4.2.[39]

Patients with band 3 Montefiore and Tuscaloosa have spherocytic haemolytic anaemia with protein 4.2 deficiency.[41,42] Both of these alleles have missense mutations in the cytoplasmic domain, but symptoms are manifest in heterozygotes with band 3 Tuscaloosa and only in the homozygote with band 3 Montefiore.

The reason for this difference is unclear. Band 3 Coimbra has a substitution in the putative second ectoplasmic loop of band 3, causing mild HS Several mutant band 3 alleles modulate the severity of the disease. Band 3 Mondeza and Montefiore, which both have missense mutations in the cytoplasmic domain, aggregate the HS symptoms of band 3 Coimbra when either is co-inherited. A normally silent promoter mutation in band 3 Genas increases the clinical severity of HS in a patient with band 3 Lyon.[33]

Since band 3 protein functions as an anion exchanger and is also expressed in the kidney cortical collecting ducks, patients with HS and band 3 deficiency have been

tested for a defect in acid-base homeostasis. Two patients with band 3 Pribrom have incomplete distal renal tubular acidosis, but most HS patients with band 3 deficiency have no evidence of metabolic acidosis.[8] In patients with band 3 compinas, there is al, increased basal urinary bicarbonate excretion but efficient urinary acidification.[36] A bovine model of band 3 deficiency exhibits only mild acidosis[24] and there are no obvious metabolic disturbances in mice that eliminates both the red cell and kidney isoforms.[8]

Band 3 mutations have been found in patients with dominant distal renal tubular acidosis, but these patients have no red cell abnormality and the mutations identified are no different form those associated with HS.[8]

The band 3 or CD233 gene is located on chromosome 17q21 – q22 and is part of the anion exchanger (AE) family. CD233 is expressed in the erythrocyte plasma membrane where it functions as a chloride/bicarbonate exchanger involved in carbon dioxide transport from tissues to lungs. The protein comprises two domains that are structurally and functionally distinct. The N-terminal 40 kDa domain is located in the cytoplasm and acts as an attachment site for the red cell skeleton by binding ankyrin, thus helping to maintain the mechanical properties and integrity of the red cell. This domain also binds a number of other red cell peripheral proteins. The glygosylated C-terminal membrane-associated domain contain 12-14 membrane spanning segments and carries out the stilbene disulphonate-sensitive exchange transport of anions. The cytoplasmic tail at the extreme C-terminus of the membrane domains binds carbonic anhydrase II. CD233 associates with the red cell membrane protein glycophorin A and this association promotes the correct folding and translocation of CD233. CD233 is predominantly dimeric but forms tetramers in the presence of ankyrin. A truncated form

of CD233 is expressed in the basolateral membrane of alpha-intercalated kidney cells and is involved in whole body pH homeostasis, because of its role in acid secretion by the distal nephron.[62]

Many CD233 mutations are known in man and these mutations can lead to hereditary spherocytosis, and defective kidney acid secretion leading to distal renal tubular acidosis. Other CD233 mutations that do not give rise to disease result in novel blood group antigens which form the Diego blood group system. CD233 knockout mice have high mortality and severe haemolytic anaemia due to unstable red cells. Naturally occurring CD233 null cattle have a similar phenotype.

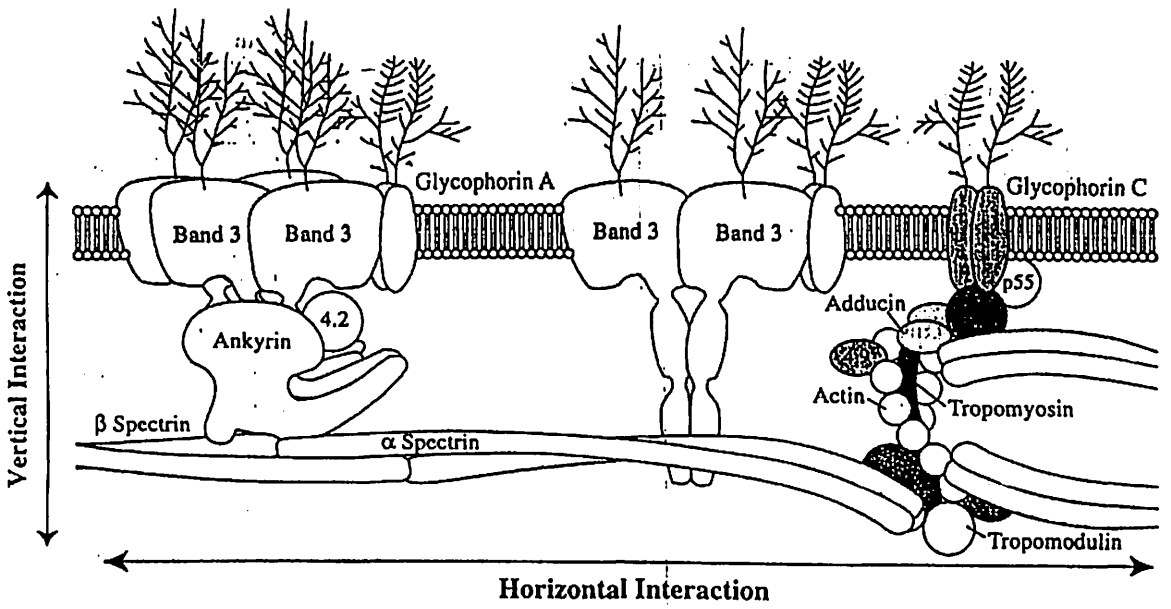

Figure 1.6 Schematic model of red cell membrane, with the vertical and horizontal interaction of its components indicated.

Estimated frequencies of mutations in different membrane proteins in HS and HE/HPP are as follows. Vertical interaction: hereditary spherocytosis: band 3, ~20%; protein 4.2, ~5%; anykrin, ~45%; β spectrin, ~30%. Horizontal interaction: hereditary elliptocytosis/ hereditary pyropoikilocytosis: β spectrin, ~5%; a spectrin, ~80%; protein 4.1, ~15%. The relative position of the various proteins is correct, but the proteins and lipids are not drawn to scale. Adapted from Lux & Palek (1995).