

Homozygous Southeast Asian ovalocytosis in five live-born neonates

Southeast Asian ovalocytosis (SAO) is an autosomal dominant inherited red blood cell (RBC) membrane disorder caused by the heterozygous deletion of codons 400–408 in SLC4A1/band 3/anion exchanger 1 (*AE1*).¹ This deletion leads to misfolding of the protein, creating an inactive anion-transporter and altering the mechanical stability of the RBC. Heterozygous SAO is characterized by the presence of stomatocytes, theta cells (RBC with two stomas), macro-ovalocytes and $\geq 25\%$ ovalocytes in the peripheral blood smears.² Although heterozygous SAO carriers are generally asymptomatic, homozygous SAO was considered to be lethal.³ However, one successful birth of a homozygous SAO individual, born to asymptomatic heterozygous SAO Comorian parents, was reported in 2014 showing an association with severe dyserythropoietic anemia.⁴ Here, we report the birth of five unrelated homozygous SAO babies in Malaysia showing that homozygous SAO is not as rare as previously thought. These babies belong to an SAO cohort collected from January 2007 to March 2020 at University Sains Malaysia (USM), which includes a total of 68 patients. The study was instigated to further understand the physiological implications of heterozygous SAO within the Malay population. The detection of numerous homozygous SAO births has highlighted the need for the development of good practice to support the survival of these babies.

SAO has a distinctive geographical distribution, occurring mostly in areas of Southeast Asia including neighboring regions of the Malay Peninsula, coastal regions of Papua New Guinea, Thailand, Indonesia, Taiwan and even Madagascar in the western Indian Ocean.^{5,6} Investigations into the high incidence of SAO in malarious areas revealed that the SAO mutation confers protection from severe malaria caused by *Plasmodium vivax* and *Plasmodium falciparum*.^{7,8}

AE1 is the most abundant RBC membrane protein and mediates the efficient transport of carbon dioxide in the blood.¹ Heterozygous SAO individuals have only 50% normal RBC anion transport activity and thus less efficient gas transport. The heterozygous expression of the SAO AE1 in the red cell membrane not only affects the folding and structure of AE1 but also affects the structure of other red cell membrane proteins depressing a relatively high number of red cell antigens.⁹ Neonatal anemia has also been reported in SAO families.²

A truncated form of AE1 is expressed in the α -intercalated cells of the kidneys and is essential for acid secretion in the urine.¹ Loss of AE1 anion-transport activity in the kidney causes distal renal tubular acidosis (dRTA) and occurs when AE1 expression is severely reduced as in hereditary spherocytosis (HS)^{10,11} or when kidney AE1 activity is severely reduced¹ or trafficking of kidney AE1 is impeded in the α -intercalated cell.¹ Many dRTA mutations are recessive. When SAO AE1 is inherited in trans to a recessive dRTA AE1 mutation then the compound heterozygote will completely lack AE1 anion transport activity. This phenomenon explains the prevalence of dRTA in South East Asia where there are a number of recessive dRTA AE1 mutations maintained in the population.¹²

Homozygous SAO individuals, with no functioning AE1, and homozygous HS individuals, with no AE1, suffer from hemolytic anemia, dRTA and failure to thrive.^{3,10,11} An in-depth study of homozygous SAO cells,¹³

showed expression of SAO AE1 during erythropoiesis (*in vitro*) affected trafficking and cytokinesis resulting in numerous multinucleated erythroblasts and reduced proliferation and enucleation. Unexpectedly, even the *in vitro* cultured heterozygous SAO erythroblasts displayed multinuclearity and reduced enucleation, with an intermediate phenotype part way between the homozygous and control cells,¹³ suggesting that heterozygous SAO individuals may have a mild dyserythropoiesis. Mature homozygous SAO RBC show signs of altered membrane composition, oxidative damage, are very large, oval and unstable.^{4,13} AE1 null HS individuals sometimes have a mild dyserythropoiesis¹⁰ and their RBC are unstable.¹¹ Extensive clinical management of AE1 null individuals, including splenectomy, has improved the survival of these children, some of whom have now reached adolescence. Similarly, the Comorian homozygous SAO child is now 10 years old and quite well, receiving regular transfusions, iron chelation and acidosis treatment.^{4,13} From Malaysia, a live-born homozygous SAO neonate with dRTA was reported in 2015 but the child failed to thrive after 2 years.¹⁴

In Malaysia, where the prevalence of SAO is around 4% within the Malay ethnic group,¹⁵ SAO diagnostic tests are performed mostly upon still birth or recurring miscarriages of the mother. Several heterozygous SAO mothers in this cohort have a history of past miscarriages and complications during pregnancy such as hydrops fetalis as well as intrauterine death (Figure 1). Furthermore, SAO was identified in 18 of 22 dRTA patients (81.8%) when the association between SAO and dRTA was studied in the Malaysian dRTA cohort.¹⁵ Here, we provide an overview of a Malaysian heterozygous SAO cohort and describe the clinical characteristics of five live-born homozygous SAO cases in Malaysia.

This SAO cohort includes a total of 107 patients whose blood samples were tested for the 27 base pair deletion of AE1 using polymerase chain reaction (PCR) and blood smears (*Online Supplementary Appendix; Online Supplementary Table S1*). Among the 107 tested patients, 68 cases were positive where 93% (63 of 68 cases) of SAO positive cases belonged to Malay ethnicity. Within this Malaysian SAO cohort, 81 cases studied belong to a total of 28 families. Among these 28, 23 families had at least one heterozygous SAO family member. The remaining 26 samples were from individual cases, of which 15 were heterozygous SAO. The majority of the SAO positive cases (43%, n=29) are from Penang, followed by Kuala Lumpur (22%, n=15), Johor (10%, n=7), Sabah (9%, n=6), Kedah (9%, n=6), and Melaka (7%, n=5) (*Online Supplementary Figure S3*). A variety of RBC morphologies were observed from peripheral blood film examinations of the SAO positive cases, which includes macro-ovalocytes, stomatocytes, theta cells, spherocytes and target cells (*Online Supplementary Table S1; Online Supplementary Figure S1*).

Surprisingly, a total of six homozygous SAO were found in our cohort, five of whom were live-born (Figure 1, Table 1, *Online Supplementary Appendix*, and *Homozygous SAO case histories*). All parents of live-born homozygous SAO cases, with the exception of one (GO 009/16), were confirmed heterozygous for the SAO AE1 deletion; case GO 012/19 was a homozygous fetus that suffered intra-uterine death at 29 weeks. All the five live-born cases were delivered prematurely, at 30 to 35 weeks of gestation. Similar to previous homozygous SAO live-borns, our five homozygous SAO live-borns suffered a severe phenotype requiring intra-uterine transfusions, post-delivery transfusions and ventilation sup-

port. Despite efforts to manage their condition, two of the five patients reported here died, one during the neonatal period and one at 2 months. Even though the other two cases survived during the neonatal period, one of them (GO 012/13) succumbed during infancy due to complications of anemia and the other is not traceable after 2 months. Further details of the obstetric history of

these families are provided in Figure 1 and the *Online Supplementary Table S2*.

Among the five live-born homozygous SAO cases, three of them had severe anemia at birth and two of them developed it at 26 weeks (GO/01/10) and 25 weeks (GO 012/13). All except GO 001/14 had hydrops fetalis and hepatosplenomegaly (Table 1). Physical examination

Table 1. Comparison of five Malaysian live-born homozygous Southeast Asian ovalocytosis (SAO) cases with a previously reported Comorian homozygous SAO case.

Case	GO 003/10	GO 010/10	GO 001/14	GO 009/16	GO 012/13 ¹⁴	Comorian SAO homozygote ^{4,13}
Gestation (weeks)	34	35	33	30	35	29
Age at diagnosis	Newborn	15 days old	3 months old	Newborn	4 months old	No data
Sex	Male	Male	Male	Male	Male	Male
Race/nationality	Malay	Malay	Malay	Malay	Malay	Comorian
Living status	Died at 6 hours of life	Died at 2 months old	Not known	Died within few hours of life	Died at 24 months old	Alive, 10 years old (2020)
Anemia	Severe anemia at birth (Hb 4.9 g/dL)	Severe anemia at 26 weeks gestation (Hb 2.4 g/dL)	Severe anemia at birth (Hb 5.0 g/dL)	Severe anemia at birth (Hb 2.0 g/dL)	Severe anemia at 25 weeks gestation (Hb 3.0 g/dL); Hb 7.4 g/dL at 4 months old	Severe anemia at 22 weeks gestation (Hb 2.9 g/dL)
Hydrops fetalis	Yes	Yes	No	Yes	Yes	Yes
Hepatosplenomegaly	Yes	Yes	No	Yes	Yes	No data
Physical examination	Pale	Bronzed	Pale, jaundiced	Very pale, born not vigorous, poor APGAR score, umbilical bleeding	Good APGAR, very pale, jaundiced, tachypnoeic	No data
Full blood picture	Many elliptocytes, stomatocytes, and NRBC. Normal platelet count. Leucoerythroblastic picture and neutrophilia	Many spherocytic RBC, crenated RBC, fragmented RBC, NRBC, and reticulocytes. Slightly reduced platelet (adequate for hemostasis, no clumping noted).	Severe anemia with marked reticulocytosis, NRBC, many spherocytes and microspherocytes. Theta cells also observed.	Spherocytosis with polychromasia. Severe anemia with marked reticulocytosis, NRBC, many spherocytes and microspherocytes. Theta cells also seen. Normal platelets.	Hypochromic microcytic cells, elliptocytes as well as teardrop cells were observed.	Rich erythroid lineage. Large ovalocytes, large reticulocytes and macrocytes. Normal myeloid and megakaryocyte lineages.
Patient genetics	Homozygous SAO deletion	Homozygous SAO deletion	Homozygous SAO deletion	Homozygous SAO deletion	Homozygous SAO deletion	Homozygous SAO deletion, β globin "La De'sirade", α thalassemia
Complication and management	1. Required intubation (ventilatory and inotropic support). 2. Packed cell transfusion after birth but died within few hours of life.	1. Intrauterine transfusion twice due to fetal anemia at 26 weeks. 2. Ventilated after birth. 3. Packed cell transfusion at day 5 of life.	1. Ventilated after birth. 2. Exchange transfusion at 5 hours of life. 3. Six episodes of hemolysis within 42 days of life (all required packed cell transfusion). 4. Hematinic prescribed.	1. Ventilated after birth.	1. Intrauterine transfusion at 25 weeks due to fetal anemia and hydrops fetalis. 2. 2-weekly transfusions from 1 month old. 3. Developed renal tubular acidosis type I due to persistent metabolic acidosis and hypokalemia.	1. Monthly transfusions since birth. 2. dRTA (3 months old) treated with oral sodium bicarbonate and potassium gluconate. 3. High ferritinemia (6 months old) treated with deferoxamine mesilate. 4. 10 years old (2020): still undergoing regular transfusions, iron chelation, and acidosis treatment.

DRTA: distal renal tubular acidosis; Hb: hemoglobin; NRBC: nucleated red blood cell; PCR: polymerase chain reaction; RBC: red blood cell; SAO: Southeast Asian ovalocytosis; APGAR score: appearance, pulse, grimace, activity, and respiration score.

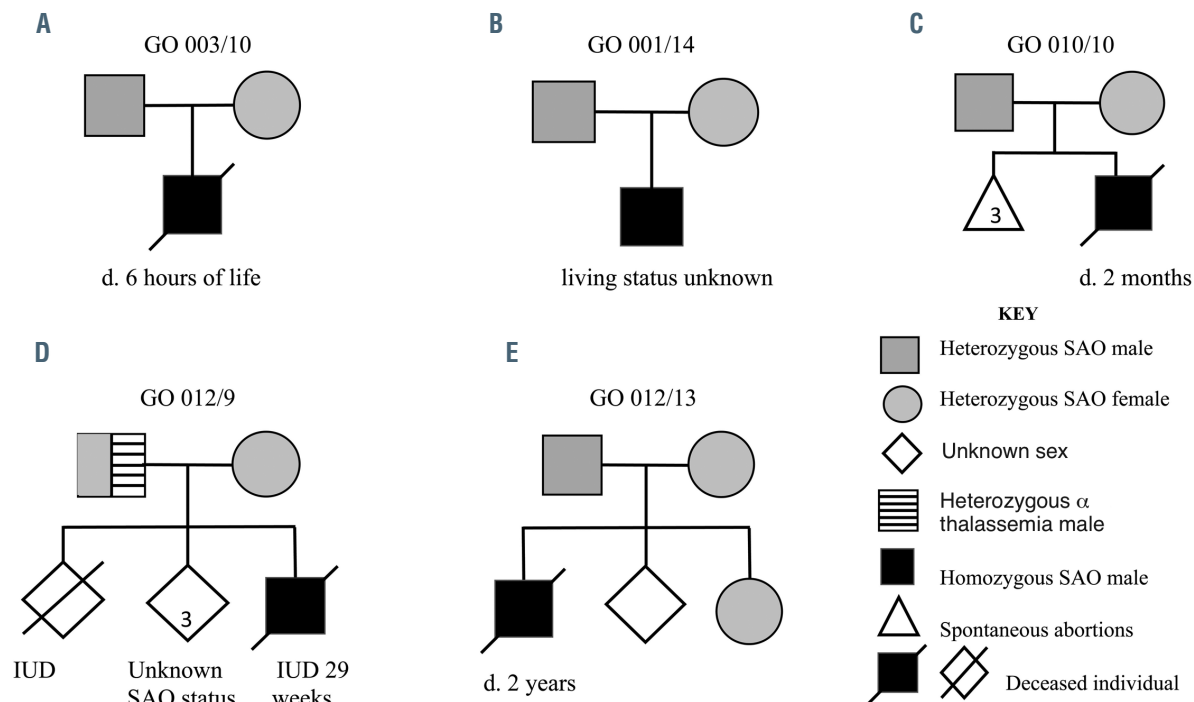


Figure 1. Family pedigree charts of five homozygous Southeast Asian ovalocytosis cases. Families of each homozygous Southeast Asian ovalocytosis (SAO) cases are represented: (A) GO 003/10, (B) GO 001/14, (C) GO 010/10, (D) GO 012/19, and (E) GO 012/13. Squares and circles denote male and females, respectively, with filled ones (grey or black) showing members affected with SAO. The numbered triangle (C) shows number of spontaneous abortions that occurred in the couple's past pregnancies. Parents of two SAO homozygous children (GO 012/19 and GO 010/10) had multiple unsuccessful pregnancies. The mother of GO 012/19 suffered two intra-uterine deaths (including GO 012/19), while the mother of GO 010/10 suffered three spontaneous abortions. None of these other unsuccessful pregnancies were investigated further. On the other hand, parents of GO 012/13 have two living children among whom one is SAO heterozygous, while parents of GO 012/19 have three living children with unknown SAO status (see the *Online Supplementary Table S2*). d: died; IUD: intra-uterine death.

revealed pale appearance, distended abdomen due to hepatosplenomegaly, and jaundice in all five cases. One of the five cases was reported to have dRTA diagnosed in the third month¹⁴ but the other four were not tested. Despite medical interventions, all five homozygous SAO children failed to thrive. This could be due to hepatosplenomegaly, severe anemia or lack of appropriate clinical management. It is difficult to gain more insights into the implications of the SAO homozygous cases as incomplete clinical data of the SAO cohort was collected, lacking data on hemoglobin abnormalities and blood group compatibility. It is also not known whether prenatal diagnosis and termination of pregnancy were offered. These services are not always available, and/or not acceptable to parents. It is now evident that careful clinical management can improve the life of homozygous SAO individuals.⁴ The long-standing assumptions that homozygous SAO is lethal, and that heterozygous SAO is asymptomatic should be re-assessed.

Several point mutations in AE1 that result in a wide range of complications have been reported, many of them leading to anemia and dRTA.^{2,12} Hemolytic anemia is recorded in children with homozygous G701D or compound heterozygous G701D/SAO, V850/SAO, A858D/SAO and V850/A858D mutations.¹ Therefore, homozygous SAO and other AE1 null individuals, who show disparity in survival, may be indicating the cumulative result of hemolysis, acidosis and other hematological abnormalities influenced by the co-occurrence of additional mutations.

To our knowledge, this preliminary Malaysian SAO cohort study, for the first-time reports five homozygous

SAO live-born cases. Since Malaysia and Southeast Asia have a high prevalence of SAO it would be beneficial to screen for SAO at antenatal clinics, at least for parents with a history of hydropic or stillborn fetuses. It is also essential to investigate the impact of heterozygous mutant SAO AE1 on other pathological conditions and ageing. Development of international and national guidelines for assessing a number of hematological, renal, and hepatic conditions in combination with genetic mutation markers to improve survival of homozygous AE1 null probands is needed.

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