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Homozygous Southeast Asian Ovalocytosis in five live-born neonates

Amanda A. Lavinya1, Ruzzieatul A. Razali2, Munirah A. Razak3, Rashidah Mohamed4, Emmanuel J. Moses5, Meera Soundararajan6, Lesley J Bruce7, Jeyanthy Eswaran1,8, and Narazah Mohd Yusoff5

1Newcastle University Medicine Malaysia, Johor, Malaysia.
2Clinical Genetics Section, Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia, Penang, Malaysia.
3Pathology Department, Hospital Kuala Lumpur, Kuala Lumpur, Malaysia.
4Pathology Department, Hospital Ampang, Selangor, Malaysia.
5Cluster of Regenerative Medicine, Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia, Penang, Malaysia.
6Department of Applied Sciences, Northumbria University, Newcastle upon Tyne, U.K.
7Bristol Institute for Transfusion Sciences, NHS Blood and Transplant, Bristol, U.K.
8Institute of Clinical and Translational Research, Faculty of Medicine, Newcastle University, Newcastle upon Tyne, U.K.

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Running title: Homozygous SAO

Corresponding authors: narazah@usm.my, Jeyanthy.Eswaran@newcastle.ac.uk

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To the Editor:

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). This deletion leads to misfolding of the protein, creating an inactive anion-transporter and altering the mechanical stability of the RBCs. Heterozygous SAO is characterized by the presence of stomatocytes, theta cells (RBCs with two stomas), macro-ovalocytes and ≥25% ovalocytes in the peripheral blood smears. Although heterozygous SAO carriers are generally asymptomatic, homozygous SAO was considered to be lethal. 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. 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 Universiti 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. 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.

AE1 is the most abundant RBC membrane protein and mediates the efficient transport of carbon dioxide in the blood. 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. Neonatal anemia has also been reported in SAO families.

A truncated form of AE1 is expressed in the alpha-intercalated cells of the kidneys and is essential for acid secretion in the urine. 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)\textsuperscript{10,11} or when kidney AE1 activity is severely reduced\textsuperscript{1} or trafficking of kidney AE1 is impeded in the alpha-intercalated cell\textsuperscript{1}. Many dRTA mutations are recessive. When SAO AE1 is inherited \textit{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\textsuperscript{12}.

Homozygous SAO individuals, with no functioning AE1, and homozygous HS individuals, with no AE1, suffer from hemolytic anemia, dRTA and failure to thrive\textsuperscript{3,10,11}. An in-depth study of homozygous SAO cells\textsuperscript{13}, showed expression of SAO AE1 during erythropoiesis (\textit{in vitro}) affected trafficking and cytokinesis resulting in numerous multinucleated erythroblasts and reduced proliferation and enucleation. Unexpectedly, even the \textit{in vitro} cultured heterozygous SAO erythroblasts displayed multinuclearity and reduced enucleation, with an intermediate phenotype part-way between the homozygous and control cells\textsuperscript{13}, suggesting that heterozygous SAO individuals may have a mild dyserythropoiesis. Mature homozygous SAO RBCs show signs of altered membrane composition, oxidative damage, are very large, oval and unstable\textsuperscript{4,13}. AE1 null HS individuals sometimes have a mild dyserythropoiesis\textsuperscript{10} and their RBCs are unstable\textsuperscript{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, with regular transfusions, iron chelation and acidosis treatment\textsuperscript{4,13}. From Malaysia, a live-born homozygous SAO neonate with dRTA was reported in 2015 but the child failed to thrive after two years\textsuperscript{14}.

In Malaysia, where the prevalence of SAO is around 4\% within the Malay ethnic group\textsuperscript{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\textsuperscript{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
Among the 107 tested patients, 68 cases were positive where 93% (63 out 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%, \( n=5 \)) (Supplement figure 3). A variety of RBC morphology were observed from peripheral blood film examination of the SAO positive cases, which includes macro-ovalocytes, stomatocytes, theta cells, spherocytes and target cells (Supplement table 1 and Supplement figure 1).

Surprisingly, a total of six homozygous SAO were found in our cohort, five of whom were live-born (Figure 1, Table 1, Supplementary file, 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 intrauterine death at 29 weeks. All the five live-born cases were delivered prematurely, in 30 to 35 weeks of gestation. Similar to previous homozygous SAO live-borns, our five homozygous SAO live-borns suffered a severe phenotype requiring intrauterine transfusions, post-delivery transfusions and ventilation support. Despite efforts to manage their condition, two of the five patients reported here died, one during the neonatal period and one at two months. Even though the other two cases survived during the neonatal period, one of them (GO 012/13) had succumbed during infancy due to complications of anemia and the other is not traceable after two months. Further detail of obstetric history of these families are provided in Figure 1 and Supplement table 2.

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

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.
References

Table 1: Comparison of five Malaysian live-born homozygous SAO cases with a previously reported Comorian homozygous SAO case.

<table>
<thead>
<tr>
<th>Case</th>
<th>GO 003/10</th>
<th>GO 010/10</th>
<th>GO 001/14</th>
<th>GO 009/16</th>
<th>GO 012/13</th>
<th>Comorian SAO homozygote&lt;br&gt;(2013)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gestation (weeks)</strong></td>
<td>34</td>
<td>35</td>
<td>33</td>
<td>30</td>
<td>35</td>
<td>29</td>
</tr>
<tr>
<td><strong>Age at diagnosis</strong></td>
<td>Newborn</td>
<td>15 days</td>
<td>3 months</td>
<td>Newborn</td>
<td>4 months</td>
<td>No data</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td><strong>Race/Nationality</strong></td>
<td>Malay</td>
<td>Malay</td>
<td>Malay</td>
<td>Malay</td>
<td>Comorian</td>
<td></td>
</tr>
<tr>
<td><strong>Living status</strong></td>
<td>Died at 6 hours of life</td>
<td>Died at 2 months old</td>
<td>Not known</td>
<td>Died within few hours of life</td>
<td>Died at 24 months old</td>
<td>Alive, 10 years old (2020)</td>
</tr>
<tr>
<td><strong>Anaemia</strong></td>
<td>Severe anaemia at birth (Hb 4.9 g/dl)</td>
<td>Severe anaemia at 26 weeks gestation (Hb 2.4 g/dl)</td>
<td>Severe anaemia at birth (Hb 5.0 g/dl)</td>
<td>Severe anaemia at birth (Hb 2.0 g/dl)</td>
<td>Severe anaemia at 25 weeks gestation (Hb 3.0 g/dl); Hb 7.4 g/dl at 4 months old</td>
<td>Severe anaemia at 22 weeks gestation (Hb 2.9 g/dl)</td>
</tr>
<tr>
<td><strong>Hydrops fetalis</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Hepatosplenomegaly</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No data</td>
</tr>
<tr>
<td><strong>Physical examination</strong></td>
<td>Pale</td>
<td>Bronzed</td>
<td>Pale, jaundiced</td>
<td>Very pale, born not vigorous, poor APGAR score, umbilical bleeding</td>
<td>Good APGAR, very pale, jaundiced, tachypnoiec</td>
<td>No data</td>
</tr>
<tr>
<td><strong>Full blood picture</strong></td>
<td>Many elliptocytes, stomatocytes, and NRBCs. Normal platelet count. Leucoerythroblastic picture and neutrophilia.</td>
<td>Many spherocytic RBC, crenated RBC, fragmented RBC, NRBCs, and reticulocytes. Slightly reduced platelet (adequate for haemostasis, no clumping noted).</td>
<td>Severe anaemia with marked reticulocytosis, NRBCs, many spherocytes and microspherocytes. Theta cells also observed.</td>
<td>Spherocytosis with polychromasia. Severe anaemia with marked reticulocytosis, NRBCs, many spherocytes and microspherocytes. Theta cells also seen. Normal platelet.</td>
<td>Hypochromic microcytic cells, elliptocytes as well as teardrop cells were observed.</td>
<td>Rich erythroid lineage. Large ovalocytes, large reticulocytes and macrocytes. Normal myeloid and megakaryocyte lineages.</td>
</tr>
<tr>
<td><strong>Patient genetics</strong></td>
<td>Homozygous SAO deletion</td>
<td>Homozygous SAO deletion</td>
<td>Homozygous SAO deletion</td>
<td>Homozygous SAO deletion</td>
<td>Homozygous SAO deletion</td>
<td>Homozygous SAO deletion, Beta globin “La De’ sirade”, Alpha thalassemia</td>
</tr>
</tbody>
</table>
### Table 1, continued

<table>
<thead>
<tr>
<th>Case</th>
<th>GO 003/10</th>
<th>GO 010/10</th>
<th>GO 001/14</th>
<th>GO 009/16</th>
<th>GO 012/13&lt;sup&gt;14&lt;/sup&gt;</th>
<th>Comorian SAO homozygote&lt;sup&gt;4,13&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Complication and management</strong></td>
<td>1. Required intubation (ventilatory and inotropic support). 2. Packed cell transfusion after birth but died within few hours of life.</td>
<td>1. Intrauterine transfusion twice due to fetal anaemia at 26 weeks. 2. Ventilated after birth. 3. Packed cell transfusion at day 5 of life.</td>
<td>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.</td>
<td>1. Ventilated after birth.</td>
<td>1. Intrauterine transfusion at 25 weeks due to fetal anaemia and hydrops fetalis. 2. 2-weekly transfusions from 1 month old. 3. Developed renal tubular acidosis type I due to persistent metabolic acidosis and hypokalaemia.</td>
<td>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.</td>
</tr>
</tbody>
</table>

dRTA, distal renal tubular acidosis; Hb, hemoglobin; NRBC, nucleated red blood cell; PCR, polymerase chain reaction; RBC, red blood cell; SAO, Southeast Asian Ovalocytosis
Figure legends

**Figure 1: Family pedigree charts of five homozygous SAO cases.** Families of each homozygous 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 intrauterine deaths (including GO 012/19), while the mother of GO 010/10 suffered 3 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 Supplement table 2). d, died; IUD, intrauterine death.
Supplementary file

1. Methods

Between January 2007 and March 2020, a total of 108 (one in duplicate) samples were tested for the Southeast Asian Ovalocytosis (SAO) 27 base pair deletion of Band 3 using polymerase chain reaction (PCR) as previously described\(^1\). Briefly, genomic DNA was extracted from whole blood. The region containing the SAO 27 base pair deletion was amplified using forward primer 5’ GGG CCC AGA TGA CCC TCT GC 3’ and reverse primer 5’ GCC GAA GGT GAT GGC GGG TG 3’. Three-step cycling was performed with initial denaturation at 94 °C for 1 minute, followed by annealing at 63.5 °C for 1 minute, and extension at 72 °C for 3 minutes. The amplification step was repeated for 30 cycles followed by a final extension at 72 °C for 7 minutes. The PCR products were separated on 2% agarose gels and visualized using ethidium bromide. Product sizes for normal and mutant Band 3 genes were expected to be 175 base pairs and 148 base pairs, respectively.

For SAO diagnosis based on peripheral blood film method, a patient is considered SAO positive when there is a presence of ≥ 25% ovalocytes and stomatocytes on blood smears\(^2,3\). Patient records containing other clinical information were obtained from respective hospitals throughout Malaysia. In total, there were 62 heterozygous SAO cases, 6 homozygous SAO cases, and 28 families that were investigated.

Ethics Statement: This study is an observation from the diagnostic cohort. Hence, fully anonymized audits are exempted from ethical review.

References:

2. Supplementary figures and table

Supplement figure 1: Representative images of peripheral blood film (PBF) using Wright’s stain with 40X magnification on patient GO 012/13 (A, B) and his parents (C, D). Blood smear on the homozygous SAO patient reveals the presence of elliptocytes, polychromasia, spherocytes/microspherocytes and small fragments pre-transfusion (A) whereas dimorphic features are seen post-transfusion (B). The PBF of both parents (C, D) show abundant macro-ovalocytes, stomatocytes, and knizocytes which are typical features of SAO.
Supplement figure 2: Representative image of SAO Band 3 mutation detection with PCR on patient GO 001/14 and his parents. The SAO mutation was analyzed by amplification of exon 11 of SLC4A1 gene by PCR followed by electrophoresis on 2% agarose gel. Sample that does not have the 27 bp deletion in Band 3 will give a distinct band of 175 bp as shown in lane 3 (L3), the normal control. Heterozygous SAO samples will give two distinct bands at 175 bp and 148 bp, whereas homozygous SAO will give one distinct band at 148 bp. Sample from patient GO 001/14 shows homozygosity (L5), while the parents are heterozygous SAO carriers (L6–L7). Lane 1 (L1), standard DNA markers; Lane 2 (L2), negative control.
Supplement figure 3: Geographical locations of cases tested positive for SAO in Malaysia between January 2007 and March 2020.
Supplement table 2: Family history of homozygous SAO cases.

<table>
<thead>
<tr>
<th>Case</th>
<th>GO 003/10</th>
<th>GO 010/10</th>
<th>GO 012/13</th>
<th>GO 001/14</th>
<th>GO 009/16</th>
<th>GO 012/19 (IUD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family genetics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>SAO heterozygote</td>
<td>SAO heterozygote</td>
<td>SAO heterozygote</td>
<td>SAO heterozygote</td>
<td>Blood film suggests SAO</td>
<td>SAO heterozygote, Alpha plus thalassemia 3.7 kb deletion heterozygote</td>
</tr>
<tr>
<td>Mother</td>
<td>SAO heterozygote</td>
<td>SAO heterozygote</td>
<td>SAO heterozygote</td>
<td>SAO heterozygote</td>
<td>Blood film suggests SAO</td>
<td>SAO heterozygote</td>
</tr>
<tr>
<td>Living children</td>
<td>None</td>
<td>None</td>
<td>1 with no SAO deletion 1 SAO heterozygote</td>
<td>1 homozygous SAO (untraceable)</td>
<td>None</td>
<td>3 living children with unknown SAO status</td>
</tr>
</tbody>
</table>

Total number of pregnancies including homozygous SAO: 1, 4, 3, 1, 1, 5
Number of deaths at infancy/neonatal period: 1, 1, 1, 0, 1, 0
Number of intrauterine deaths including homozygous SAO: 0, 0, 0, 0, 0, 2
Number of spontaneous abortions: 0, 3, 0, 0, 0, 0

Abbreviations: IUD, intrauterine death; SAO, Southeast Asian Ovalocytosis
3. **Homozygous SAO case histories**

**Patient GO 003/10**

Patient GO 003/10 was a newborn Malay male delivered at 34 weeks of gestation by emergency caesarean section due to fetal distress. After birth, he required intubation and was admitted into the Intensive Care Unit with ventilatory and inotropic support. He appeared pale, had hepatosplenomegaly, and was also edematous while his chest X-ray results showed that he had a globular shaped heart. Full blood picture examination revealed the presence of abundant elliptocytes, stomatocytes, as well as nucleated red blood cells (NRBC). The patient also had neutrophilia and showed evidence of leucoerythroblastic picture as characterized by presence of numerous myelocytes and NRBCs. In addition, he had a low Hb concentration (4.9 g/dl; Normal range: 14–22 g/dl). Platelet count, on the other hand, was unremarkable. As such, the patient was diagnosed with hydrops fetalis secondary to hereditary stomatocytic-elliptocytosis and hemolysis; homozygous SAO was confirmed upon PCR analysis. Packed red cell transfusion was administered to the newborn, however, the patient succumbed to the condition at six hours of life. In terms of family history, both parents were tested heterozygous for the 27 base pair deletion in Band 3. Also, their peripheral blood film examination showed typical SAO red blood cell morphology characterized by the presence of many elliptocytes and stomatocytes. Hence, both parents were diagnosed with hereditary stomatocytic-elliptocytosis with the father having mild leukocytosis. There was no history of consanguinity and patient GO 003/10 was the couple’s first born child.

**Results from laboratory investigations of patient GO 003/10**

- **Liver Function Test**
  - Total bilirubin : 114 µmol/l (raised) – jaundiced
  - Total protein (Normal: 66–83) : 59 g/l (reduced)
  - Albumin (Normal: 35–52) : 23 g/l (reduced)
  - Globulin (Normal: 23–35) : 3 g/l (reduced)
  - ALT (Normal: <35) : 51 U/L (raised)
  - ALP (Normal: 30–120) : 217 U/L (raised)
- **Calcium (Normal: 2.20–2.65)** : 3.0 mmol/l (slightly raised)
- **LDH (Normal: 140–280)** : 13418 U/L (raised) – hemolysis
**Patient GO 010/10**

Patient GO 010/10 was a 15 day old Malay male born at 35 weeks of gestation by emergency caesarean section due to fetal distress. At 26 weeks of gestation, fetal scan and doppler revealed an enlarged heart with pericardial effusions, oligohydramnios, and features of fetal anemia. Fetal anemia was confirmed when cordocentesis test showed low Hb concentration of 2.4 g/dl. Fetal transfusion *in utero* was then performed twice. After birth, the patient appeared bronzed, had large hepatomegaly (4–5 cm) and a palpable spleen (1 cm), which are signs of hydrops fetalis. The patient also suffered from respiratory distress and required ventilation. Peripheral blood film examination revealed RBCs that were either spherocytic, crenated, fragmented, or nucleated. Presence of reticulocytes were also observed while white blood cell count was unremarkable. However, he had slightly reduced platelet count but no clumping was observed. The patient had suffered from ongoing non-immune hemolytic anemia with jaundice. Exchange transfusion was performed at 2 days old followed by packed red cell transfusion at 5 days old, after which hemolysis had improved. Unfortunately, he succumbed to his condition at 2 months of age. Both the patient’s parents were tested heterozygous for the 27 base pair deletion in Band 3. Features of hereditary ovalostomatocytosis were also observed in their peripheral blood film examination. Interestingly, the mother had previously suffered three spontaneous abortions with a history of antenatal anemia; neither of the cases were fully investigated. Patient GO 010/10 was the first live-born child for the couple.

**Patient GO 012/13**

A detailed history of patient GO 012/13 has been reported by Asnawi et al., 2015. The patient was the first born for his parents. He failed to thrive with the condition and suffered developmental delay. He succumbed to his condition at 2 years of age. The parents subsequently had two other children; one with no SAO deletion in Band 3 and the other is SAO heterozygous. Both siblings are growing well with no evidence of hydrops fetalis or anemia.

**Patient GO 001/14**

Patient GO 001/14 was a 3 month old Malay male born prematurely at 33 weeks of gestation via spontaneous vaginal delivery. At birth, he weighed only 1.36 kg. He was
severely pale and had signs of respiratory distress, which required ventilatory support. He was extubated at 18 hours of life and weaned to room air at day 4 of life. Physical examination had shown no dysmorphism, deformities, hepatosplenomegaly, features of hydrops, or cutaneous bleed. He did however have low Hb levels (5.0 g/dl) at birth. Packed cell transfusion was performed at 5 hours after birth and later he developed gross hematuria at 10 hours of life. Brain ultrasound showed bilateral grade I-II intraventricular hemorrhage. He was severely jaundiced with total bilirubin of 259–322 μmmol/l and LDH of 7482 U/L and hence required exchange transfusion. Within 42 days of life, he had suffered six episodes of hemolysis which required packed cell transfusions. His hemoglobin ranged between 8.5–9.3 g/dl and achieved 12.0 g/dl post-transfusion. The baseline hemoglobin was accepted between 8.0–9.0g/dl. Hematinic was commenced and continued. However, at day 55 and 85 days of life, his hemoglobin dropped again to 5.6 g/dl and 5.9 g/dl, respectively. Full blood picture of both of his parents showed ovalostomatocytes with some macro-ovalocytes and theta cells. Sample from the infant showed hemolytic picture with spherocytosis, nucleated RBCs, and ovalostomatocytes. Coomb’s test excluded the possibility of the infant having hereditary spherocytosis. Upon PCR analysis, the parents were subsequently confirmed heterozygous carriers of SAO while the child was homozygous.

Results from laboratory investigations of patient GO 001/14

- Mean corpuscular volume (MCV): 143.9 fl
- Reticulocyte: 12.9%
- White cell count: 29.8
- Platelet count: 242
- G6PD: Normal.
- Kleihauer test, direct Coomb test, renal profile and coagulation profile was normal.
- Liver Function Test revealed increasing trend of direct hyperbilirubinemia.

Patient GO 009/16

Patient GO 009/16 was a newborn Malay male born prematurely at 30 weeks of gestation. At birth, the infant was very pale, had distended abdomen, was not vigorous, and had poor
APGAR score. The infant showed signs of hydrops fetalis and also suffered bleeding from the umbilicus. The infant required ventilatory support after birth. Unfortunately, the infant succumbed to the condition a few hours after delivery. Peripheral blood film examination on the patient sample showed spherocytosis with polychromasia, while the same examination was suggestive of SAO for both parents. However, SAO status of both parents was not confirmed with PCR.

**Patient GO 012/19**

Patient GO 012/19 is a case of intrauterine death at 29 weeks with the fetus showing signs of hydrops fetalis. The patient was male and of Malay ancestry. Both parents were tested heterozygous for SAO Band 3 deletion, with the father also being heterozygous for alpha plus thalassemia 3.7 kb deletion. This patient is the second intrauterine death for the couple. They have three living children, all of which have unknown SAO status as the children were not screened.

**Supplement table legend**

**Supplement table 1:** The clinical information of patients tested for 27 base pair deletion in Band 3 between January 2007- March 2020. In the table the abbreviations: "/", denotes presence; bp, base pair; D, days; F, female; IUD, intrauterine death; M, male; NA, not available; NB, newborn; NRBC, nucleated red blood cells; PBF, peripheral blood film; RBC, red blood cells; SAO, Southeast Asian Ovalocytosis; SB, stillbirth; U, unknown; Y, years