Homozygous Southeast Asian ovalocytosis in five live-born neonates

Amanda A. Lavinya,[1] Ruzzieatul A. Razali,[2] Munirah A. Razak,[3] Rashidah Mohamed,[4] Emmanuel J. Moses,[5] Meera Soundararajan,[6] Lesley J. Bruce,[7] Jeyanthy Eswaran[1,8] and Narazah Mohd Yusoff[5]

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

Correspondence: NARAZAH MOHD YUSOFF - narazah@usm.my

JEYANTHY ESWARAN - jeyanthy.eswaran@newcastle.ac.uk

doi:10.3324/haematol.2020.268581

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 ℃ for 1 minute, followed by annealing at 63.5 ℃ for 1 minute, and extension at 72 ℃ for 3 minutes. The amplification step was repeated for 30 cycles followed by a final extension at 72 ℃ 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.

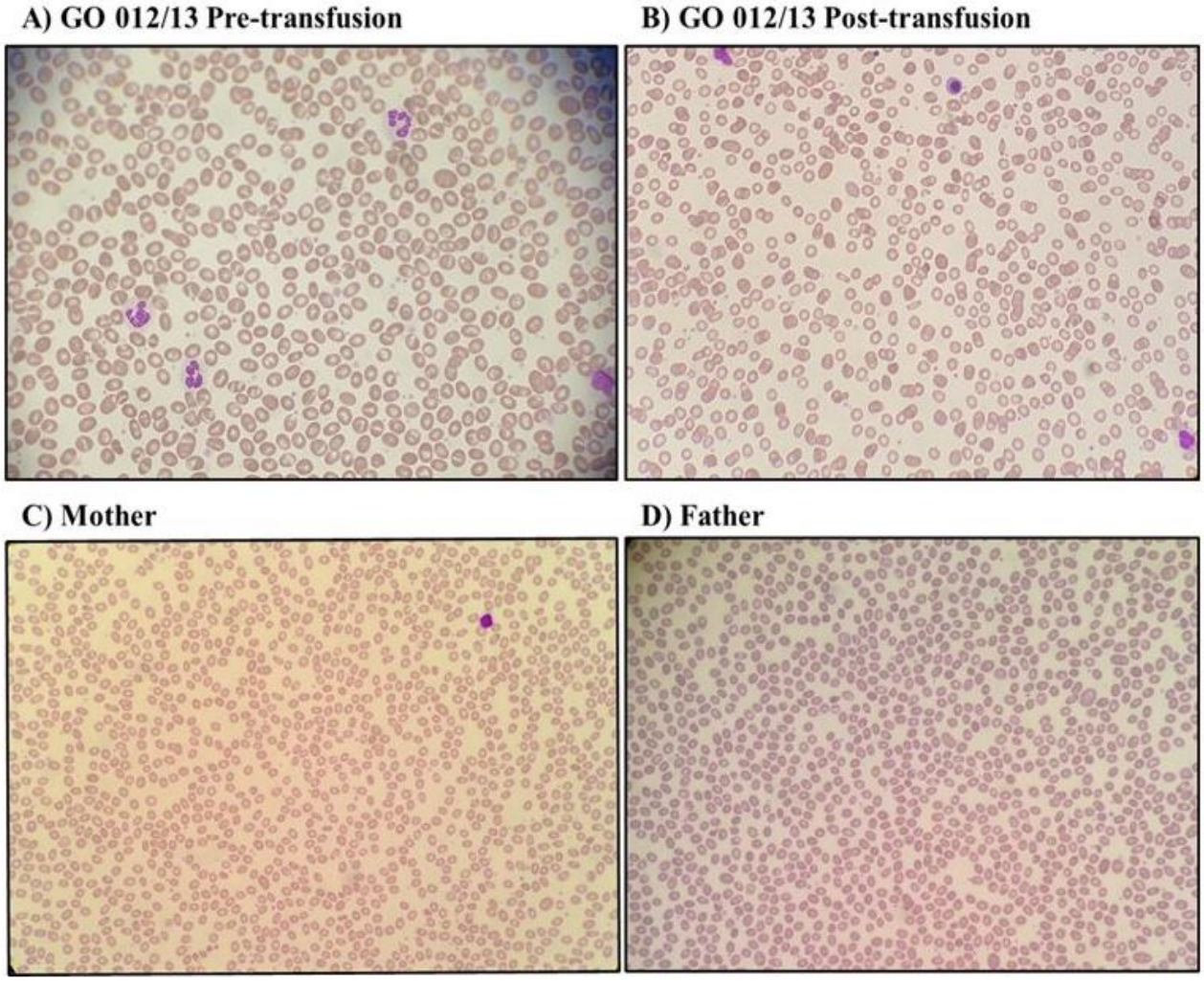
For SAO diagnosis based on peripheral blood film method, a patient is considered SAO positive when there is a presence of ≥ 25% ovalocytes and stomatocytes on blood smears[2,3]. Patient records containing other clinical information were obtained from respective hospitals throughout Malaysia. In total, 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.

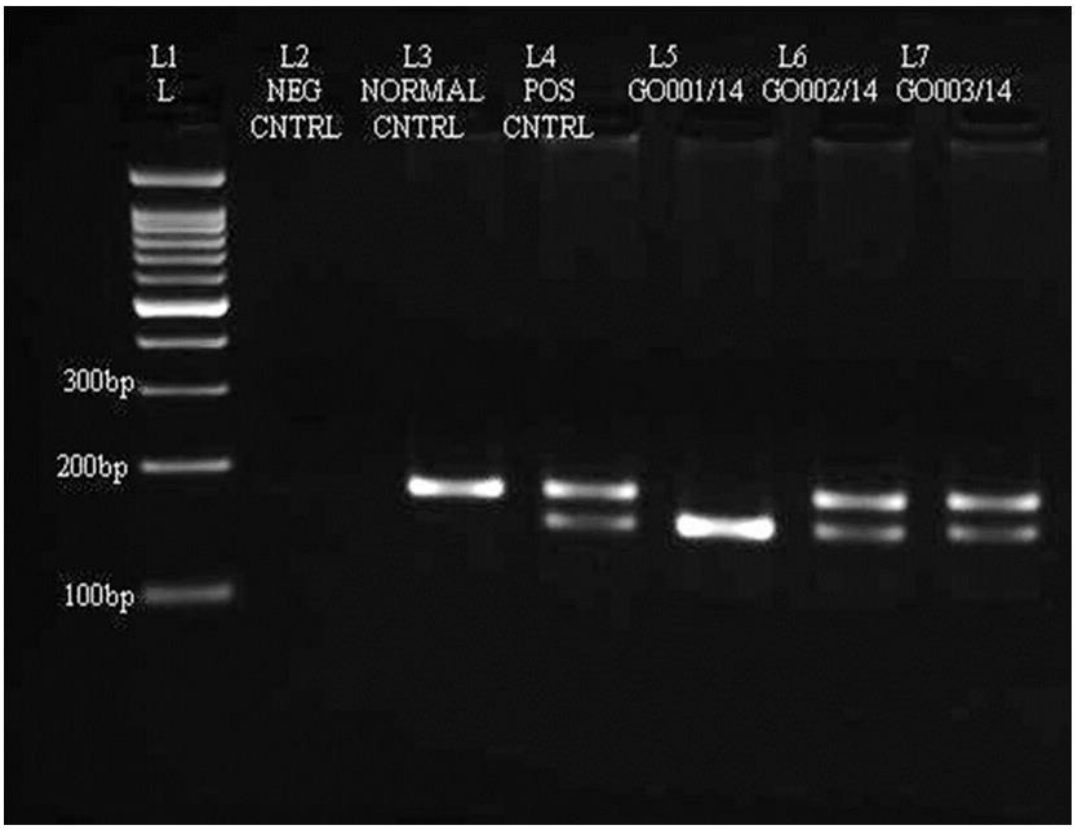
References:

1. Yusoff NM, Van Rostenberghe H, Shirakawa T, Nishiyama K, Amin N, Darus Z, et al. High prevalence of Southeast Asian ovalocytosis in Malays with distal renal tubular acidosis. J Hum Genet. 2003;48(12):650-653.
2. Liu SC, Zhai S, Palek J, Golan DE, Amato D, Hassan K, et al. Molecular defect of the band 3 protein in southeast Asian ovalocytosis. N Engl J Med. 1990; 323(20):1530-1538.
3. Raman Y, Saleem M, Ahmad R, and Yusoff NM. Coinheritance of Southeast Asian ovalocytosis and the β-thalassemia trait in a Malay family. Asian Biomedicine. 2017;9(5):691-695.

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.

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

<u>Results from laboratory investigations of patient GO 003/10</u>

- 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