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High prevalence of Southeast Asian ovalocytosis in Malays with distal renal tubular acidosis

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Abstract Southeast Asian ovalocytosis (SAO) is a red blood cell abnormality common in malaria-endemic regions and caused by a 27 nt deletion of the band 3 protein gene. Since band 3 protein, also known as anion exchanger 1, is expressed in renal distal tubules, the incidence of SAO was examined in distal renal tubular acidosis (dRTA) in Malays in Kelantan, Malaysia. Twenty-two patients with dRTA and 50 healthy volunteers were examined for complication of SAO by both morphological and genetic analyses. SAO was identified in 18 of the 22 dRTA patients (81.8%), but only two of the 50 controls (4%). The incidence of SAO was significantly high in those with dRTA ($p < 0.001$), indicating a dysfunctional role for band 3 protein/anion exchanger 1 in the development of dRTA.

Keywords dRTA · SAO · Malays · Band 3 protein/anion exchanger

Introduction

Southeast Asian ovalocytosis (SAO) is an autosomal dominant red blood cell abnormality common in Southeast Asian countries (Lie-Injo 1965; Takeshima et al. 1994; Serjeantson et al. 1997). The underlying molecular defect of SAO is a 27-nt deletion in exon 11 of the band 3 protein/anion exchanger 1 gene on chromosome 17 (Jarolim et al. 1991; Mohandas et al. 1992), which leads to the deletion of nine amino acids (codons 400–408) of the boundary between the cytoplasmic and the transmembrane domains of the gene product. SAO is characterized by increased red cell rigidity and provides a likely barrier against malaria infection (Mohandas et al. 1984). SAO has been considered hematologically asymptomatic (Takeshima et al. 1994), though SAO is identified in neonatal hyperbilirubinemia (Laosombat et al. 1999).

Distal renal tubular acidosis (dRTA) is characterized by an inability to generate a normal minimum urinary pH even in the presence of severe systemic acidosis. It results from the impaired secretion of hydrogen ions from the distal nephron. The consequence is metabolic acidosis, often with nephrocalcinosis, hypokalemia and metabolic bone disease. Defects in several enzymes or transporters involving transepithelial acid secretion and bicarbonate reabsorption in alpha-intercalated cells cause dRTA (Karet 2002).

Band 3 protein/anion exchanger 1 is a bicarbonate/chloride exchanger expressed not only in red blood cell membranes, but also in the basolateral membrane of collecting tubule alpha-intercalated cells (Kollert-Jons et al. 1993; Tanner 1997). It is reasonable to consider that SAO and dRTA occur together. There have been several reports of dRTA complicated with SAO but this co-occurrence was initially assumed to be random (Thong et al. 1997; Kaitwatcharachai et al. 1999). To determine the association between dRTA and SAO in the Malay patients in Kelantan, Malaysia, the incidence of SAO was analyzed in 22 racial Malays with dRTA. Here the incidence of SAO was shown to be significantly high in those

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with dRTA, indicating a dysfunctional role for band 3 protein/anion exchanger 1 in the development of dRTA.

Subjects and methods

The records of patients diagnosed with dRTA in the University Sains Malaysia (USM) Hospital, Kelantan, Malaysia were retrieved. Kelantan, the northeast state of peninsular Malaysia, is a predominantly rural area with a mainly homogeneous Malay population. This population is well suited to genetic studies in that it exhibits linguistic isolation with little immigration and has many large, stable families. In the USM hospital, renal tubular acidosis in adults is found in 3.5 patients per year on average, with dRTA being the most common type (Zainal 1994).

The diagnosis of dRTA was made based on an inability to lower the urine pH below 6.0 in the presence of systemic acidosis (Clague and Krause 1997). Only racial Malays (children and adults) confirmed to have primary dRTA were included in the study. Patients with other renal or systemic diseases were excluded. Between January 1990 and August 1999, there were 22 patients who fulfilled the criteria, and all were included in the study. Thirteen were unrelated adults and nine were children (Table 1). Among the children, there were three pairs of siblings from three unrelated families. Family histories gave evidence of neither consanguinity nor relationships between families.

In children, the diagnosis of dRTA was usually made by 5 years of age, and six out of nine patients were female. The children presented with failure to thrive (9/9) accompanying commonly with rickets (7/9) (Table 1). Most had body weights and heights below the third percentile. All had severe metabolic acidosis, alkaline urine, and hypokalemia at presentation. The majority had nephrocalcinosis (7/9). Most dRTA children were anemic, with hemoglobin values below 10.8 g/dl (not shown), and one of the children had anemia with a hemoglobin value of 8.8 g/dl. The three pairs of siblings were included.

For the adults, the triad of muscle weakness, hypokalemia, and systemic metabolic acidosis were the characteristic features at

presentation, and a normal serum alkaline phosphatase level and skeletal X-rays were noted. Plasma bicarbonate, when it could be measured, was decreased. In cases where acidosis was not severe and urine pH was equivocal, acid loading tests were performed as described (Wrong and Davies 1959) for confirmation of the diagnosis of dRTA (5/13). Most patients also had moderate to severe hypokalemia and hyperchloremia at diagnosis, and some had documented nephrocalcinosis as shown by ultrasound. The hemoglobin values of all the adults were within normal limits. Ten out of 13 patients were female.

Informed consent was obtained from all patients and 50 healthy Malay adult volunteers with no history of dRTA. Venous blood (2 ml) was collected from all subjects and controls in sterile EDTA tubes. A peripheral blood film was prepared and stained with Wright's stain. A red cell morphological study was done, and the percentage of ovalocytes was recorded. A diagnosis of ovalocytosis was made if there were 25% or more ovalocytes in the peripheral blood film (Palek and Lambert 1990).

Genomic DNA was isolated from whole blood, and a region encompassing the 27 nt deletion was amplified by polymerase chain reaction (PCR) as previously described (Jarolim et al. 1991; Mohandas et al. 1992; Takeshima et al. 1994) using the forward primer 5'-GGGCCAGATGACCCTCTGC-3' for bases 1098-1117 and the reverse primer 3'-GCCGAAGGTGATGGCGGGTG-5' for bases 1272-125. Initial denaturation was done at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 3 min, with a final extension at 72°C for 7 min. The amplification was performed for 30 cycles. The PCR products were size-fractionated in 2% agarose gels and visualized by ethidium bromide staining. The expected sizes of the PCR products were 175 bp and 148 bp for the normal and mutant genes, respectively. The Fisher exact test was used to determine the *p* value. A *p* value of less than 0.05 was considered significant.

Results

SAO was screened by peripheral blood film examination. Eighteen out of 22 dRTA patients showed

Table 1 Data for cases of distal renal tubular acidosis. Values of serum K⁺ (normal: 3.5–5.0), serum Cl⁻ (normal: 98–107) and plasma HCO₃⁻ (normal: 20–30) are in millimole per liter. Normal range of urine pH is 7.36–7.44. SAO Southeast Asian ovalocytosis, F female, M male, Nt not tested, + present, - not present, NA not available

Case	Gender/age (years)	Serum K ⁺	Serum Cl ⁻	Plasma HCO ₃ ⁻	Urine pH	Failure to thrive	Rickets	Nephrocalcinosis	SAO morphological	SAO genetic
1	F/0.5	2.4	NA	10.9	7	+	-	-	+	+
2	F/0.5	1.6	113	4.8	7.5	+	-	-	+	+
3 ^a	M/1	2.4	114	14.1	7	+	+	+	+	+
4 ^a	F/1.8	1.4	90	5.5	7.5	+	+	+	+	+
5 ^b	F/3	2.6	118	11	7	+	+	+	+	+
6 ^c	F/4	2.9	112	13.5	7.5	+	+	+	+	+
7 ^b	F/5	1.6	NA	NA	6.5	+	+	+	+	+
8	M/7	2.4	116	8.3	7	+	+	+	+	+
9 ^c	M/7	3.1	115	14.9	7.5	+	+	+	+	+
10	F/20	2.8	115	16.5	6.5	-	-	Nt	+	+
11	F/24	1.2	110	7.6	7	-	-	Nt	+	+
12	F/27	3	114	16.2	7	-	-	Nt	+	+
13	F/28	2	113	16	7.5	-	-	Nt	+	+
14	F/28	1.9	109	17	7	-	-	Nt	+	+
15	M/30	3.1	NA	14.2	6	-	-	Nt	+	+
16	F/30	3	NA	13	6.5	-	-	Nt	Normal	-
17	F/38	1.9	130	12.8	6.5	-	-	+	+	+
18	F/39	1.8	111	16.2	7.5	-	-	Nt	Normal	-
19	F/40	1.8	115	11.4	7	-	-	Nt	+	+
20	M/45	3.3	NA	23.8	6	-	-	+	+	+
21	F/47	1.6	103	26.9	7.5	-	-	Nt	Normal	-
22	M/63	1.2	NA	16	6.5	-	-	Nt	Normal	-

a, b, c Three pairs of siblings from three different families

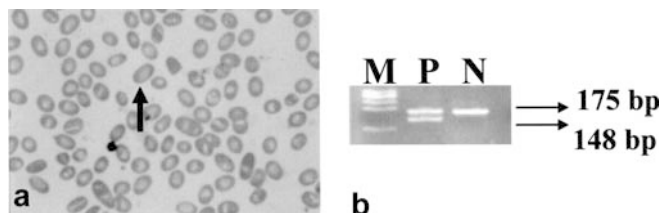


Fig. 1 **a** Morphological finding of Southeast Asian ovalocytosis (SAO): Ovalocytotic changes are observed in more than 25% of red blood cells in peripheral blood smear. *Arrow* indicates a representative ovalocyte. **b** PCR amplification products of the band 3 protein/anion exchanger 1 gene: Two amplified products corresponding to 175 bp and 148 bp were obtained from all samples of patients diagnosed with SAO (*P*) but only one product was found in normal controls (*N*). *M.P* and *N* refer to a size maker, patient and normal, respectively

ovalocytosis (Fig. 1). In the control group, however, only two out of 50 individuals were detected to have SAO. To confirm SAO, PCR amplification of the exon 11 encompassing region of the band 3 protein/anion exchanger 1 gene was done. All DNA samples from those morphologically diagnosed with ovalocytosis had two amplified products: one normal sized product and the other a small product harboring the 27-nt deletion (Fig. 1). Samples from those not diagnosed with ovalocytosis had only the normal sized product. Therefore, it was concluded that SAO was present in 18 out of 22 dRTA patients (81.8%). In contrast, only two of the 50 healthy controls (4%) were confirmed to have SAO. There was a significant difference in the incidence of SAO between the two groups ($p < 0.001$) (Fig. 2). This difference remained highly significant ($p < 0.001$), even after the exclusion of the second member of the same family.

Discussion

This study screened SAO not only in dRTA patients but also in healthy adults. Remarkably, it was disclosed that 4% of control adults in Kelantan, northeast of peninsular Malaysia had SAO (Fig. 2). Though the incidence of SAO seemed very high, it is still in the expected range corresponding to previously unpublished data from Kelantan (5%) (Yusoff 1994, personal communication) and the report from Indonesia where the incidence of SAO is 12.6% (Kimura et al. 2002). SAO has been reported in Malay populations from different countries (Lie-Injo, 1965; Takeshima et al. 1994; Alimsardjono et al. 1997; Serjeantson et al. 1997). The high prevalence of SAO is due to the result of natural selection by malaria infection, since SAO provides defense against malaria. Accordingly, Kelantan has long been an endemic region for malaria, and there are still several rural areas with a high incidence of *Plasmodium falciparum* infection, which can be complicated by cerebral malaria.

This is the first study of the incidence of SAO in individuals with dRTA. Remarkably, SAO was shown to

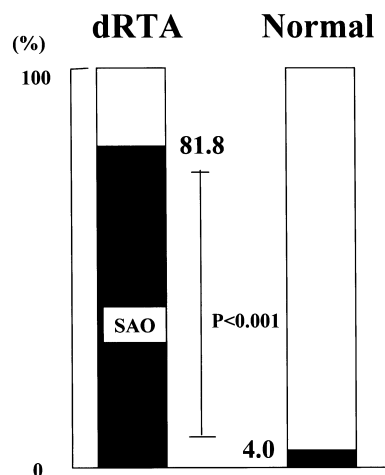


Fig. 2 Incidence of Southeast Asian ovalocytosis (SAO) in patients with distal renal tubular acidosis (dRTA) is significantly high compared to normal adults

be highly prevalent in dRTA patients of the Malay race (Fig. 2). This is in contrast to findings from Western countries; defects in several enzymes or transporters involved in transepithelial acid secretion and bicarbonate reabsorption in alpha-intercalated cells have been shown to cause dRTA (Karet 2002). dRTA can be familial, with autosomal dominance the most common form of inheritance. X-linked, autosomal recessive, and sporadic cases have been reported (Karet 2002). Recently, mutations in the band 3 protein/anion exchanger 1 gene have been found in an autosomal dominant form of dRTA of European origin (Karet et al. 1998).

The fact that more than 80% of dRTA patients had SAO indicated that SAO is a predisposing factor for the development of dRTA. The association of dRTA and SAO is understandable, considering that band 3 protein/anion exchanger 1 is expressed in both red blood cells and renal distal tubules. However, it is difficult to directly correlate the two, because almost all of the SAO patients have been reported to be asymptomatic not only hematologically but also nephrologically (Takeshima et al. 1994). Therefore, it seemed that one mutation in the band 3 protein/anion exchanger 1 gene is not sufficient to develop dRTA.

Recently, some studies of dRTA have indicated a strong association between SAO and dRTA, suggesting a common genetic basis for both SAO and dRTA (Vasuvattakul et al. 1999; Bruce et al. 2000; Yenchit-somanus et al. 2002). Among our patients, more females were affected than males (Table 1). Three familial cases were limited to siblings, but no transmission from generation to generation was identified. Furthermore, four out of 22 dRTA cases did not involve SAO. Altogether it seems difficult to pinpoint the cause of dRTA in our cases at this moment.

In our previous study, SAO was shown to be linked with one missense mutation changing AAG to GAG of codon 56 (Takeshima et al. 1994). Therefore, it is highly possible that other mutations are present outside the

region of the band 3 protein/anion exchanger 1 gene examined here. In fact, the combination of the classical mutation for SAO with other band 3 protein/anion exchanger 1 mutations has only recently been discovered (Bruce and Tanner 1999). The anion transport-inactive SAO allele behaves as a band 3 null allele so that normally recessive band 3 protein/anion exchanger 1 mutations associated with dRTA are expressed as pseudodominants (Vasuvattakul et al. 1999; Bruce et al. 2000; Yenchitsomanus et al. 2002). Thus, the samples from the dRTA subjects of this study will be analyzed further with the aim of identifying a second band 3 protein/anion exchanger 1 mutation in the gene. A longitudinal follow-up study of a cohort of our asymptomatic SAO patients is ongoing.

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