Ovalocytosis without band 3 gene 27-bp deletion and malaria infection

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Abstract Genetic adaptation to malaria is a longstanding research topic in anthropology and human genetics. Southeast Asian ovalocytosis (SAO) has been documented to have resistance to malaria; however, the implications of existing data are controversial. In particular, SAO resistance to malaria is unclear in terms of the types of SAO, with/without the 27-base pair deletion in the band 3 gene (B3Δ27). To shed light on the relationships between SAO and malaria, we surveyed *Plasmodium* infection, erythrocyte morphology, and the presence of the B3Δ27 among the residents of a selected area in East Kalimantan, Indonesia where malaria is endemic. We screened peripheral blood smears ($n = 128$) for *Plasmodium* infection and erythrocyte morphology under a microscope, and then DNA was extracted from the smears to be used as a template for a polymerase chain reaction to detect the B3Δ27. The prevalence of infection with *Plasmodium* including *Plasmodium vivax* and *Plasmodium falciparum* was approximately 30%. Among a total of 128 subjects, 9.4% and 18.0% showed moderate (ovalocytic cells: 30–49%) and severe (ovalocytic cells: 50–100%) ovalocytosis, respectively. A total of three B3Δ27 carriers were identified. The data set was statistically analyzed and we observed that (1) higher ovalocytic rate resulted in lower malaria infection and (2) the B3Δ27 did not prevent malaria infection. These results suggest that resistance of SAO to malaria is due to SAO without the B3Δ27.

Key words: Southeast Asian ovalocytosis (SAO), band 3 (AE1) gene, Indonesia, malaria

Introduction

Genetic adaptation to malaria is a longstanding research topic in anthropology and human genetics. Certain red blood cell (RBC) defects are suggested to have resistance to malaria. RBC defects, including those with altered RBC shape, have been reported from several malaria-endemic regions. The distribution of the defects meets the hypothesis that subsets of the RBC defects became prevalent through natural selection by malaria. The most famous example of such malaria-resistant RBC defects is sickle cell anemia.

RBC deformity is another example of such RBC defects in the Asian-Pacific region where ovalocytosis has commonly been recorded. Among several types of hereditary ovalocytosis recorded to date, Southeast Asian ovalocytosis (SAO) is characterized by the oval shape of red blood cells and is clinically asymptomatic. This type of hereditary ovalocytosis has been observed widely in Southeast Asia and Melanesia and the prevalence is as high as 50% in some populations (Lie-injo, 1965; Amato and Booth, 1977). Serjeantson et al. (1977) hypothesized that SAO is resistant to malaria because the distribution of SAO coincides with

malaria endemic areas. The higher frequencies of SAO found in adults than in the young of the same population in malaria endemic areas (Baer et al., 1976) also supported this hypothesis and suggested a selective advantage of SAO in such areas. However, several studies on malaria resistance of SAO are controversial in terms of parasite density or *Plasmodium* species (Baer et al., 1976; Serjeantson et al., 1977; Cattani et al., 1987; Foo et al., 1992).

A 27-bp deletion in the band 3 gene [Band3, EPB3, AE1: solute carrier family 4 member 1 (SLC4A1), OMIM+109270-0002] found among individuals with SAO (Jarolim et al., 1991) was considered the underlying molecular basis for SAO; however, the 27-bp deletion in the band 3 gene (B3Δ27) did not account for all SAO cases (Tanner et al., 1991). Moreover, low frequencies of the B3Δ27 in Southeast Asians were demonstrated (Kimura et al., 1998) and the presence of other molecular mechanism(s) for SAO is thus postulated. The diagnosis of SAO used to be based on erythrocyte morphology with poorly standardized criteria; relationships between *Plasmodium* infection and SAO have been promiscuous probably due to these unsystematic criteria. After the discovery of the B3Δ27, relationships between SAO caused by the B3Δ27 (B3Δ27-SAO) and malaria resistance were studied and severity of malarial disease symptoms was shown to subside in individuals with B3Δ27-SAO (Allen et al., 1999). However, the resistance of SAO to malaria disease is still unclear in terms of the types of SAO,

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with/without the B3Δ27, as well as *Plasmodium* species. Our previous study showed that the B3Δ27 did not alter the parasite infection rate or the species-specific prevalence (Kimura et al., 2002; Shimizu et al., 2005).

To shed light on relationships between ovalocytosis and malaria, we have screened for malaria infection, erythrocyte morphology, and the B3Δ27 in East Kalimantan, Indonesia.

Materials and Methods

A survey was carried out in Tanjung Redep, East Kalimantan, Indonesia (Figure 1), where malaria was endemic. The residents were of mixed population with different ethnic origins. Among them, Berau was the major and indigenous tribe in the region followed by other tribal groups such as Manado, Toraja, and Bugis who had migrated from northern, central, and southern Sulawesi Island, respectively. A total of 128 individuals (age range: 1–63 years; mean age: 28 years) (Table 1) were recruited and blood specimens were collected with oral informed consent.

Thick and thin blood smears were prepared and stained with Giemsa for microscopic examination. The presence of malaria parasites and the frequency of ovalocytic cells were determined by an experienced hematologist. According to the frequency of ovalocytic cells observed on the blood smears, subjects were categorized into three groups (fre-

Figure 1. Location of the survey area.

Table 1. Ovalocytosis and malaria infection in each group in Tanjung Redep, East Kalimantan, Indonesia

Group	n	Group of ovalocytosis			Plasmodium ¹		
		L	M	H	v.	f.	v. and f.
Berau	79	53	10	16 ³	11	5	6
Bugis	21	18	0	3	5	1	1
Manado	9	6	2 ⁴	1	0	0	0
Toraja	9	7	0	2	3	2	0
Others ²	10	9	0	1	2	2	0
Total	128	93	12	23	21	10	7

¹ v.: *vivax*; f.: *falciparum*.

² Others: Javanese (n = 4), Batak (n = 2), Dayak (n = 2) and Timor ethnic groups (n = 2).

³ Two individuals had the B3Δ27.

⁴ One individual had the B3Δ27.

quency of ovalocytic cells): group L (0–29%), group M (30–49%), and group H (50–100%) (Cattani et al., 1987; Mgone et al., 1998). Genomic DNA was extracted from blood films with the standard phenol/chloroform method. The B3Δ27 was studied by a polymerase chain reaction (PCR) method with a specific primer set (5'-GGGCCCAGATGAC-CCTCTTGC-3' and 5'-GCCGAAGGTGATGGCGGGTG-3') spanning the 27-bp deletion (Jarolim et al., 1991). An initial denaturation at 95°C for 9 minutes was followed by 40 cycles of denaturation at 94°C for 1 minute and annealing and extension at 70°C for 1 minute with a final extension at 70°C for 5 minutes. The wild type and the deletion type allele was identified by the size of the PCR product corresponding to 175 bp and 148 bp, respectively. PCR for glycoporphin C (GPC) exon 3 was carried out as described by Patel et al. (2001) for those ovalocytosis without the B3Δ27. The PCR products were subjected to electrophoresis on 4% agarose gel and visualized with ethidium bromide.

Results and Discussion

Of a total of 128 blood smears, 72.6%, 9.4%, and 18.0% was categorized into groups L, M, and H, respectively (Table 1). The B3Δ27 was found only in three subjects (2.3%) who belonged to the Berau (n = 2; group H) and the Manado (n = 1; group M). This low frequency of the B3Δ27 is consistent with the previous report (Kimura et al., 1998), and highlights the high prevalence of ovalocytosis that is not caused by the B3Δ27.

The prevalence of infection with *Plasmodium vivax* (*P. vivax*), *P. falciparum* and both species was 7.8%, 16.4%, and 5.5%, respectively. The frequency of ovalocytic cells and malaria infection was negatively correlated (Cochran-Armitage's trend test, $P = 0.0046$) (Figure 2). The prevalence of malaria infection in group H was significantly lower when compared with the rest of the population (namely groups L and M) (Fisher's exact test, $P = 0.0038$); this result was significant irrespective of inclusion or exclusion of the B3Δ27 cases (n = 3). As for the parasite species, resistance to both *P. falciparum* and *P. vivax* was demonstrated; however, resistance to *P. vivax* was more obvious. In an attempt to minimize confounding effects by heterogeneous genetic backgrounds, only the Berau ethnic group (n = 79) was then subjected to the previous analysis, and a negative correlation was also observed (Cochran-Armitage's trend test, $P = 0.007$).

We have observed the following two relationships between ovalocytosis and malaria in this study: 1) higher ovalocytic rate results in lower malaria infection, and 2) ovalocytosis without B3Δ27 may have protective effects against malaria. These observations may necessitate a reevaluation of the hypothetical implications of SAO to malaria resistance. There have been several controversial reports on the resistance of SAO against malaria. Recent studies on the relationships between malaria and the B3Δ27 revealed that B3Δ27-SAO provides protection against cerebral malaria but does not affect infection rate and density of either *P. falciparum* or *P. vivax* (Genton et al., 1995; Allen et al., 1999; Kimura et al., 2002; Shimizu et al., 2005). Morphologically identified SAO was thought to have preventive

Figure 2. Frequency of ovalocytic cells and malaria infection. Groups L, M, and H in the text correspond to 0–29%, 30–49%, and 50–100% ovalocytes, respectively.

features such as resistance to high density of parasitemia, and decreased rates of infection by *P. vivax*, *P. malariae*, and *P. falciparum* (Baer, 1976; Serjeantson, 1977; Cattani et al., 1987; Foo et al., 1992); however, this category of SAO comprised B3Δ27-SA0 and other type(s) of SAO. Because the B3Δ27 did not lead to resistance to *Plasmodium* infection, it is conceivable that the reduced *Plasmodium* infection observed in the previous microscopic studies was due to SAO without the B3Δ27.

Mgone et al. (1998) indicated that microscopic diagnosis of ovalocytosis can be influenced by the inter- and intra-variation on the part of the observer. As the B3Δ27-SA0 displays a wide range of ovalocytic phenotype, the results of past reports should be reconsidered on the presumption that microscopically diagnosed SAO comprises several molecular bases. In fact, the presence of two distinctive types of SAO, with and without the B3Δ27 (Cattani et al., 1987; Tanner et al., 1991), has been reported in Papua New Guinea. One of the molecular bases for such SAO without the B3Δ27 in Papua New Guinea was recently revealed as a deletion of GPC exon 3; the frequency and intensity of blood-stage *P. falciparum* or *P. vivax* infection was not associated with the GPC genotype (Patel et al., 2001). Concerning the ovalocytosis observed in the eastern part of Borneo Island in this study, the presence of the reported deletion in the GPC gene was not confirmed by PCR, which suggests the presence of other molecular mechanism(s) for this SAO with protective effect on malaria infection.

The controversial implications of the malaria resistance of SAO in earlier studies can be attributed to the admixture of molecularly heterogeneous SAO. In the present study, SAO without the B3Δ27 is clearly shown to contribute to lowering malaria infection rates. As it is fully expected that SAO identified microscopically cannot be attributed to a single molecular basis as shown in the previous and present stud-

ies, the existence of malaria-resistant SAO without the B3Δ27 awaits the identification of the underlying molecular bases.

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