Original Article

Microsatellite Polymorphism in the Heme Oxygenase-1 Gene Promoter Is Associated with Susceptibility to Cerebral Malaria in Myanmar

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SUMMARY: Cerebral malaria (CM) is a serious complication of *Plasmodium falciparum* malaria, and its pathogenesis leading to coma remains unknown. Heme oxygenase-1 (HO-1) catalyzes heme breakdown, eventually generating bilirubin, iron and carbon monoxide. The HO-1 gene promoter contains a polymorphic $(GT)_n$ repeat which may influence the expression level of HO-1. To explore the correlation between this $(GT)_n$ polymorphism and susceptibility to CM, we analyzed the frequencies of the $(GT)_n$ alleles in 120 Myanmarese patients with uncomplicated malaria (UM) and 30 patients with CM. The frequency of homozygotes for the short $(GT)_n$ alleles (<28 repeats) in CM patients was significantly higher than those in UM patinets ($P < 0.008$, OR = 3.14). Thus, short $(GT)_n$ alleles represent a genetic risk factor for CM.

INTRODUCTION

Cerebral malaria (CM) is the most serious complication of malaria caused by *Plasmodium falciparum*, manifesting as coma. CM may be related to the sequestration of infected red blood cells (RBCs) to the cerebral microvasculature (1,2). The adherence of parasitized RBCs to the vascular endothelium may result in local ischemia and hypoxia, microhemorrhage and the release of hemoglobin after hemolysis of parasitized erythrocytes (1-3), leading to CM in certain susceptible individuals. However, in addition to the effects of direct parasite sequestration, the possible role of host and parasite metabolic processes in inducing coma has begun to attract considerable interest.

Patients with malaria must cope with excess amounts of cellular and plasma hemoglobin, because a large number of infected and uninfected RBCs are destroyed in the spleen or rupture in the blood stream. Free hemoglobin can serve as a biological Fenton's reagent to provide iron for the generation of hydroxyl radicals (4,5), and is therefore quickly removed from circulation by haptoglobin and taken up by macrophages. Within a cell, hemoglobin is dissociated into heme and globin, and the heme moiety is cleaved to generate iron, carbon monoxide (CO) and biliverdin by heme oxygenase-1 (HO-1), a microsomal inducible enzyme (6). Biliverdin is subsequently reduced to bilirubin, a potent antioxidant (7). The potential beneficial and harmful effects of

heme breakdown products in falciparum malaria have been reviewed in a previous study (8).

In the present paper, we analyzed $(GT)_n$ repeat polymorphism in the promoter region of the inducible HO-1 of patients with CM and with uncomplicated malaria (UM), finding a significant association between homozygotes of short alleles and CM.

MATERIALS AND METHODS

Subjects: One hundred and twenty Karen patients with UM (mean age, 23.4 ± 8.1; mean hemoglobin, 11.9 ± 2.4 g/ dl) from the Mae Sot Malaria Clinic and 30 patients with CM (mean age, 23.4 ± 8.1; mean hemoglobin, 11.8 ± 3.0 g/dl) admitted to the Mae Sot General Hospital, Mae Sot, Thailand were recruited for this study between 1996 and 1997. UM was defined by a positive smear without any of the following criteria of severe malaria (3): high parasitemia (>100,000 parasites/μl), severe anemia (hematocrit <20%, or hemoglobin <7.0 g/dl) or coma., CM was diagnosed by coma without any other recognized cause of altered consciousness. The protocol, including the informed consent form, was approved by the ethical review committee of the Ministry of Health of Thailand and by the institutional review board in Nagasaki. All procedures were appropriately explained to patients in their own language.

Extraction of genomic DNA: Genomic DNA was extracted from 1 ml of peripheral venous blood with 0.05 M EDTA using a DNA extractor kit (Wako Pure Chemicals, Osaka, Japan).

Microsatellite polymorphism: To amplify the $(GT)_n$ microsatellite located at position -270 of the HO-1 gene (9), a fluorescein-conjugated 5′ primer (HeOp-1/6-FAM 5′-

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agagcctgcagcttctcaga-3′) and an unlabeled 3′ primer (HeOp-1/R 5′-acaaagtctggccataggac-3′) were used for PCR amplification with 35 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 3 min. An amplicon of at least 148 bp from positions -165 to -312 of the HO-1 gene was obtained (10). The size of the PCR products was analyzed with a laser-based automated DNA sequencer, ABI GeneScan GS-310 (Applied Biosystems, Foster City, Calif., USA), with five cloned alleles that were labeled with different colors as size markers. The repeat numbers of these cloned alleles used as size markers were 21, 23, 25, 28 and 30.

Statistical analysis: The associations between disease groups and specific classes of alleles, and between disease groups and genotype groups were analyzed for significance by the chi-square test of independence by 2×2 contingency table and the P values were corrected using Bonferroni's method. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to assess the relative disease risk conferred by a particular allele and genotype. The age and sex ratios of subjects with UM and CM were matched in the present study. Furthermore, all subject were Myanmarese, who represent a distinct ethnic population.

RESULTS

The number of $(GT)_n$ repeats in the HO-1 gene was between 14 and 38 in all subjects. The distribution of the numbers of $(GT)_n$ repeats was trimodal, with peaks at 23, 30 and 36 repeats (Figure 1). We therefore grouped the alleles into three subclasses: short (S) alleles with 14-27 repeats, intermediate (M) with 28-33 repeats and long (L) with 34-38 repeats, as described in the analysis by McGinnis and Spielman (11). The frequencies of the L, M and S allele groups were 0.11, 0.45 and 0.44, respectively, in UM patients and 0.05, 0.32 and 0.63 in CM patients. The allele frequencies in CM and UM were not significantly different.

The frequencies of the genotypes (LL, ML, MM, SL, SM, SS) of $(GT)_n$ repeats were 0.0, 12.5, 16.7, 9.2, 44.2 and
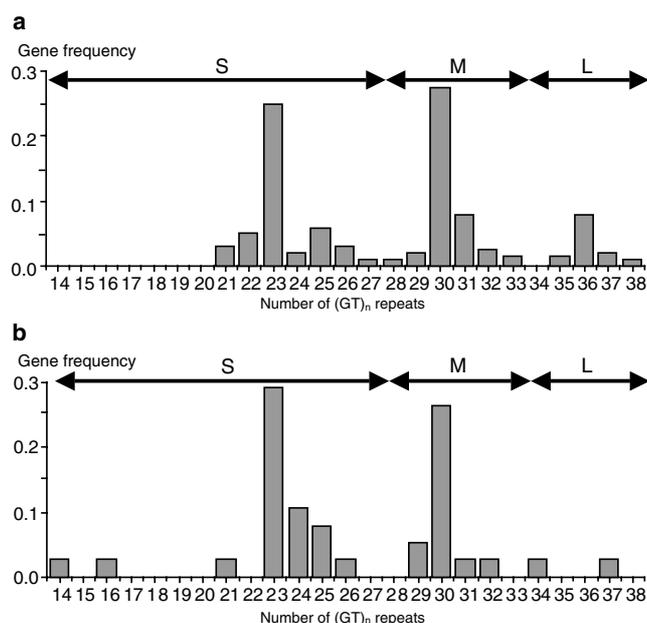
Table 1. Comparison of the genotype frequencies between uncomplicated and cerebral malaria groups

a. Genotype frequencies

Genotype	UM $n = 120$ (%)	CM $n = 30$ (%)
M/L	15 (12.5)	0 (0.0)
M/M	20 (16.7)	4 (13.3)
S/L	11 (9.2)	3 (10.0)
S/M	53 (44.2)	11 (36.7)
S/S	21 (17.5)	12 (40.0)

b. Chi-square test for S/S homozygotes in the two patients groups

Genotype	UM $n = 120$ (%)	CM $n = 30$ (%)	P value	OR	95% CI
S/S	21 (17.5)	12 (40.0)	0.008	3.14	1.32-7.49

The frequencies of the S/M/L HO-1 promoter $(GT)_n$ genotypes between cerebral malaria (CM) and uncomplicated malaria (UM).
Alleles were determined by their GT repeat numbers. L, 34-38 repeat; M, 28-33; and S, 14-27. Genotype was determined by individual combination of the alleles. SS genotype frequencies were significantly deviated between two groups.

17.5%, respectively, in UM cases ($n = 120$), and 0.0, 0.0, 13.3, 10.0, 36.7 and 40.0% in CM cases ($n = 30$) (Table 1a). The frequency of homozygotes for S alleles in the CM group (40%) was significantly higher than that in the UM group (17.5%) after Bonferroni correction ($P < 0.008$; OR = 3.14; 95% CI: 1.32-7.49) (Table 1b).

DISCUSSION

The pathogenesis of CM is complex and multifactorial. Previous studies indicate that tumor necrosis factor (TNF)-α promoter polymorphism is one of the most frequently reported candidate host factors associated with CM in Africa as well as in Asia (12-14). In addition to TNF-α, many other genes have been postulated to be involved in the pathogenesis of CM (15-18). The present study is the first to suggest an association between the length of the $(GT)_n$ repeat in the HO-1 gene promoter and clinical presentation of malaria. Individuals with genes carrying the short repeat were found to be more likely to present with CM, while those carrying the longer repeats were marginally more likely to fall into the UM category. Chen et al. (19) reported that subjects with the short allele containing $(GT)_n = 22$ showed 8 times higher transcriptional activity than those with the $(GT)_n = 30$ allele sequence, as determined by luciferase assay using rat aortic smooth muscle cells. Therefore, a short $(GT)_n$ in the promoter may directly enhance the transcription of HO-1 in malaria patients and the resultant reaction products, CO, iron and bilirubin, may increase in the brain lesion. Recently, it was reported that HO-1 protein levels are increased in Durck's granuloma, a typical lesion of advanced CM (20). Clark et al. (21) also reported a more detailed immuno-histochemical study on the expression of inducible HO-1 protein using many CM autopsy cases in which monocytes or macrophages in the brain and lung and the Kupffer cells in the liver systemically expressed HO-1. It is not clear what induced HO-1 or how it relates to pathology, however, excess amounts of catalytic product CO might impair neuronal activity as a toxic gas or might disrupt the complex neuronal network as a neural messenger (23,24). Additionally, there have been a number of reports suggesting the toxic effect of free iron in patients with CM. Gordeuk et al. reported that iron chelation

Fig. 1. Gene frequency of the $(GT)_n$ alleles in each group.
a, Uncomplicated malaria patients ($n = 120$); b, Cerebral malaria patients ($n = 30$).

therapy enhances recovery from deep coma in CM (24) and that increased transferrin saturation may be associated with delayed recovery from coma during standard therapy (25). These findings suggest that the excess free iron directly contributes to the pathogenesis of CM.

In chronic inflammatory diseases, short alleles are less associated with severe pathology, while in the present study they were significantly associated with CM, a severe manifestation of infection. Yamada et al. reported that short $(GT)_n$ alleles are protective against smoking-induced emphysema (26) and restenosis after percutaneous transluminal angioplasty in arterial occlusive diseases (27). They showed that long $(GT)_n$ repeats reduced the level of HO-1 induction by oxidative stress in cultured cells and that short $(GT)_n$ alleles are likely to provide anti-inflammatory activities of heme breakdown products (26). It is therefore conceivable that short $(GT)_n$ alleles might provide older individuals with protection against chronic inflammatory processes. In malaria, it has not yet been determined how HO-1 is involved in the pathogenesis of CM and what tissues are involved in this HO-1 related event. Furthermore, regulation of promoter activity by the length of a microsatellite may differ between tissues. This may explain why the resistant short allele in lung and vascular diseases is susceptible to CM, (26,27) though there exists the possibility that unknown causative single nucleotide polymorphisms (SNPs) are present in linkage disequilibrium with this microsatellite allele.

Transcription of the HO-1 gene is under the regulation of the fine tuning system that includes the Bach-1 repressor (28) and $(GT)_n$ spacer polymorphism. To confirm our findings, further studies are needed, using larger populations and different ethnic groups, and examining the linkage between genotype and phenotype. Nevertheless, we can say here that the repression of HO-1 expression by pharmacological means, such as competitive inhibitors of HO-1, might provide a useful adjunct to the treatment of CM (29).

REFERENCES

1. Aikawa, M. (1988): Human cerebral malaria. Am. J. Trop. Med. Hyg., 39, 3-10.
2. MacPherson, G. G., Warrell, M. J., Looareesuwan, S. and Warrell, D. A. (1985): Human cerebral malaria: a quantitative ultrastructural analysis of parasitised erythrocyte sequestration. Am. J. Pathol., 119, 385-401.
3. World Health Organization, Communicable Diseases Cluster (2000): Severe falciparum malaria. Trans. R. Soc. Trop. Med. Hyg., 94 (Suppl. 1), S1-90.
4. Sadrzadeh, S. M. H., Anderson, O. K., Panter, S. S., Hallaway, P. E. and Eaton, J. W. (1987): Hemoglobin potentiates central nervous system damage. J. Clin. Invest., 79, 662-664.
5. Sadrzadeh, S. M. H., Graf, E., Panter, S. S., Hallaway, P. E. and Eaton, J. W. (1984): Hemoglobin: a biologic Fenton reagent. J. Biol. Chem., 259, 14354-14356.
6. Tenhunen, R., Marver, H. S. and Schmid, R. (1970): The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. J. Lab. Clin. Med., 75, 410-421.
7. Stocker. R., Yamamoto, Y., McDonagh, A. F., Glazer, A. M. and Ames, B. N. (1987): Bilirubin is an antioxidant of possible physiological significance. Science, 235, 1043-1046.
8. Shibahara, S., Kitamuro, T. and Takahashi, K. (2002): Heme degradation and human disease: diversity is the soul of life. Antiox. Redo. Signal., 4, 593-602.
9. Shibahara, S., Sato, M., Muller, R. M. and Yoshida, T. (1989): Structural organization of the human heme oxygenase gene and the function of its promoter. Eur. J. Biochem., 179, 557-563.
10. Kimpara, T., Takeda, A., Watanabe, K., Itoyama, Y., Ikawa, S., Watanabe, M., Arai, H., Sasaki, H., Higuchi, S., Okita, N., Takase, S., Saito, H., Takahashi, K. and Shibahara, S. (1997): Microsatellite polymorphism in the human heme oxygenase-1 gene promoter and its application in association studies with Alzheimer and Parkinson's disease. Hum. Genet., 100, 145-147.
11. McGinnis, R. E. and Spielman, R. S. (1995): Insulin gene 5′ flanking polymorphism length of class 1 alleles in number of repeat units. Diabetes, 44, 1296-1302.
12. Knight, J. C., Udalova, I., Hill, A. V. S., Greenwood, B. M., Peshu, N., Marsh, K. and Kwiatkowski, D. (1999): A polymorphism that affect OCT-1 binding to the TNF promoter region is associated with severe malaria. Nat. Genet., 22, 145-150.
13. McGuire, W., Hill, A. V. S., Allsopp, C. E. M., Greenwood, B. M. and Kwiatkowski, D. (1994): Variation in the TNF-α promoter region associated with susceptibility to cerebral malaria. Nature, 371, 508-511.
14. Ubalee, R., Suzuki, F., Kikuchi, M., Tasanor, O., Wattanagoon, Y., Ruangweerayut, R., Na-Bangchang, K., Karbwang, J., Kimura, A., Itoh, K., Kanda, T. and Hirayama, K. (2001): Strong association of a tumor necrosis factor-α promoter allele with cerebral malaria in Myanmar. Tissue Antigens, 58, 408-410.
15. Quaye, K. E., Ekuban, F. A., Goka, B. Q., Adabayeri, V., Kurtzhals, J. A. L., Gyan, B., Ankrah, N-A., Hviid, L. and Akanmori, B. D. (2000): Haptoglobin1-1 is associated with susceptibility to severe *Plasmodium falciparum* malaria. Trans. R. Soc. Trop. Med. Hyg., 94, 216-219.
16. Aitman, T. J., Cooper, L. D., Norsworthy, P. J., Wahid, F. N., Gray, J. K., Curtis, B. R., Mckeigue, P. M., Kwiatkowski, D., Greenwood, B. M., Snow, R.W., Hill, A. V. and Scott, J. (2000): Malaria susceptibility and CD36 mutation. Nature, 405, 1015-1016.
17. Kwiatkowski, D. (2000): Genetic susceptibility to malaria getting complex. Curr. Opin. Genet. Develop., 10, 320-324.
18. Kikuchi, M., Looareesuwan, S., Ubalee, R., Tasanor, O., Suzuki, F., Wattanagoon, Y., Na-Bangchang, K., Kimura, A., Aikawa, M. and Hirayama, K. (2001): Association of adhesion molecule PECAM-1/CD31 polymorphism with susceptibility to cerebral malaria in Thais. Parasitol.

Int., 50, 235-239.

19. Chen, Y. H., Lin, S. J., Lin, M. W., Tsai, H. L., Kuo, S. S., Chen, J. W., Charng, M. J., Wu, T. C., Chen, L. C., Ding, P. Y., Pan, W. H., Jou, Y. S. and Chau, L. Y. (2002): Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. Hum. Genet., 111, 1-8.

20. Schluesener, H. J., Kremsner, P. G. and Meyermann, R. (2001): Heme oxygenase-1 in lesions of human cerebral malaria. Acta Neuropathol., 101, 65-68.

21. Clark, I. A., Awburn, M. M., Harper, C. G., Liomba, N. G. and Molyneux, M. E. (2003): Induction of HO-1 in tissue macrophages and monocytes in fatal falciparum malaria and sepsis. Malaria J., 2, 41-54.

22. Stevens, C. F. and Wang, Y. (1993): Reversal of long-term potentiation by inhibitors of heme oxygenase. Nature, 364, 147-149.

23. Zhuo, M., Small, S. A., Kandel, E. R. and Hawkins, R. D. (1993): Nitric oxideand carbon monoxide produce activity-dependent long-term synaptic enhancement in hippocampus. Science, 260, 1946-1950.

24. Gordeuk, V., Thuma, P., Brittenham, G., McLaren, C., Parry, D., Backenstose, A., Biemba, G., Msiska, R., Holmes, L., McKinley, E., Vargas, L., Gilkeson, R. and Poltera, A. A. (1992): Effect of iron chelation therapy on recovery from deep coma in children with cerebral malaria. New Engl. J. Med., 327, 1473-1477.

25. Gordeuk, V. R., Thumam P. E., McLarenm, C. E., Biemba, G., Zulu, S., Poltera, A. A., Askin, J. E. and Brittenham, G. M. (1995): Transferrin saturation and recovery from coma in cerebral malaria. Blood, 85, 3297-3301.

26. Yamada, N., Yamaya, M., Okinaga, S., Nakayama, K., Sekizawa, K., Shibahara, S. and Sasaki, H. (2000): Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. Am. J. Hum. Genet., 66, 187-195.

27. Exner, M., Schillinger, M., Minar, E., Mlekusch, W., Schlerka, G., Haumer, M., Mannhalter, C. and Wagner, O. (2001): Heme oxygenase-1 gene promoter microsatellite polymorphism is associated with restenosis after percutaneous transluminal angioplasty. J. Endovasc. Ther., 8, 433-440.

28. Kitamuro, T., Takahashi, K., Ogawa, K., Udono-Fujimori, R., Takeda, K., Furuyama, K., Nakayama, M., Sun, J., Fujita, H., Hida, W., Hattori, T., Shirato, K., Igarashi, K. and Shibahara, S. (2003): Bach1 functions as a hypoxia-inducible represser for the heme oxygenase-1 gene in human cells. J. Biol. Chem., 278, 9125-9133.

29. Sassa, S. (1997): Inhibition of carbon monoxide production by tin-protoporphyrin. J. Pediatr. Gastroenterol. Nutr. 6, 167-171.