A CCTTT microsatellite repeat in the inducible nitric oxide synthase (iNOS) promoter was analyzed among 256 adult patients with severe Plasmodium falciparum malaria and 179 adult patients with mild malaria living in northwestern Thailand. Genotypes with longer forms of the CCTTT repeat (alleles of ≥15 repeats) were significantly associated with severe malaria (odds ratio [OR], 2.14; \( P = .0029 \), \( \chi^2 \) test). More interestingly, the summed repeat number of both microsatellite alleles in an individual was found to be a significant risk factor for severe malaria (OR, 1.11; logistic regression analysis, \( P = .0041 \)). The single nucleotide substitution, −954G→C, in the iNOS promoter was rare in Thai patients with malaria. No variations were detected in the iNOS promoter region containing functional NF-κB elements at −5.2, −5.5, −5.8, and −6.1 kb upstream of the iNOS transcriptional start site. Thus, a CCTTT repeat in the iNOS promoter may play a key role in the pathogenesis of severe malaria.

A number of studies have shown that nitric oxide (NO) provides protection against Plasmodium falciparum in vitro [1, 2] and in rodent models of malaria [3]. In humans, NO is synthesized by 3 different NO synthases (NOSs) that convert l-arginine to l-citrulline and NO. The most important NOS in human immune response to P. falciparum is the inducible NOS (iNOS), a cytosolic Ca\(^{2+}\)-independent enzyme. iNOS is rapidly expressed in response to proinflammatory or immunological stimuli, thereby producing a large quantity of NO that can inhibit the growth of malaria parasites. NO appears to provide protection against P. falciparum in humans [4], and it is also hypothesized that NO acts as a mediator in cerebral malaria [5], which is the most severe complication of P. falciparum malaria and causes approximately 1 million deaths annually. Synthesis of NO by iNOS has been considered to affect neuronal functions and to cause coma in patients with cerebral malaria. In fact, iNOS expression was reported to increase in brains of patients with fatal cerebral malaria [6]. Because these observations suggest the importance of iNOS expression in the pathogenesis of severe malaria, it is interesting to examine whether the severity of malaria could be influenced by the polymorphisms in the iNOS promoter.

Recently, 2 polymorphisms in the iNOS promoter, a single nucleotide substitution from G to C at position −954 (−954G→C) and a pentanucleotide CCTTT repeat, were found to be associated with the severity of malaria in African populations [7, 8]. The CCTTT microsatellite polymorphism is located −2.5 kb upstream of the iNOS transcriptional start site. Kun et al. [7] showed an association of the −954C allele with protection against severe malaria in Gabonese children. Burgner et al. [8] demonstrated that shorter forms of CCTTT repeat (alleles of <11 repeats) in the iNOS promoter were associated with fatal cerebral malaria in Gambian children. These results imply that the polymorphisms in the iNOS promoter may influence the iNOS transcription level in children with malaria and, thereby, influence the clinical outcome. However, it has never been examined whether these polymorphisms show a significant association with the severity of malaria in populations other than African populations. The same association might not be found in different populations because of differences in their genetic backgrounds. To address this question, we analyzed −954G→C and CCTTT microsatellite polymorphisms in adult Thai patients with malaria.

There are functional NF-κB elements at −5.2, −5.5, −5.8, and −6.1 kb upstream of the iNOS transcriptional start site [9]. Mutations in these sites are expected to decrease the iNOS promoter activity. Thus, variation screening of the region containing these elements was also performed in this study.
Methods

Patients. A total of 256 adult patients with severe \textit{P. falciparum} malaria (152 patients with noncerebral severe malaria and 104 patients with cerebral malaria) and 179 adult patients with mild malaria (who served as control subjects) living in northwestern Thailand were enrolled in this study. All of them underwent treatment at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Clinical manifestations of malaria were classified according to the definitions and associated criteria published by the World Health Organization [10]. Cerebral malaria was defined as unrousable coma; positive results of \textit{falciparum} parasitemia caused coma were excluded. Severe malaria (noncerebral severe malaria) was defined by 1 of the following signs: high parasitemia (≤100,000 parasites/mL), hypoglycemia (glucose level <22 mmol/L), severe anemia (hematocrit <20% or hemoglobin level <7.0 g/dL), or increased serum level of creatinine (>3.0 mg/dL). Mild malaria was characterized by a positive blood smear and fever without other causes of infections and had no manifestations of severe malaria as described above. All individuals were ≥13 years of age, and the mean age for patients with severe malaria and patients with mild malaria both were 25.5 years.

DNA extraction. Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp blood kit (Qiagen).

Genotyping of the –954G→C polymorphism. The single nucleotide polymorphism in the \textit{iNOS} promoter region, –954G→C, was analyzed by direct sequencing using a 5′ primer –954F, 5′-TGTGGCTAACCCTTGTAACTC-3′ and a 3′ primer –954R, 5′-TCTCGTCATGTCAACTCT-3′. The polymerase chain reaction (PCR) products of 75 samples randomly chosen from patients with severe malaria and patients with mild malaria were used for direct sequencing with an ABI PRISM 3100 Genetic Analyzer (PerkinElmer Applied Biosystems).

Genotyping of \textit{CCTTT} microsatellite repeat polymorphism. Genotyping of the pentanucleotide repeat, \textit{(CCTTT)}\textit{n}, in the \textit{iNOS} promoter was performed by PCR with the primers designed by Xu et al. [11] and by electrophoresis with an ABI PRISM 377 Genetic Analyzer (PerkinElmer Applied Biosystems). PCR fragments were sized by Genescan software (version 2.1; PerkinElmer Applied Biosystems), as described in the manufacturer’s manual.

Variation screening of the \textit{iNOS} promoter region containing \textit{NF-κB} elements at –5.2, –5.5, –5.8, and –6.1 kb. The \textit{iNOS} promoter region containing \textit{NF-κB} elements at –5.2, –5.5, –5.8, and –6.1 kb was analyzed by direct sequencing using 2 sets of primers: 1 set, a 5′ primer \textit{NF-κB} (–5.2, –5.5)F, 5′-ACTTTCCTCATTCCCCATCCTG-3′ and a 3′ primer \textit{NF-κB} (–5.2, –5.5)R, 5′-AGGGCGTGAGTCACACAAAT-3′, was used for \textit{NF-κB} elements at –5.2 and –5.5 kb, and another set, a 5′ primer \textit{NF-κB} (–5.8, –6.1)F, 5′-TCCAAGAGCATCAAGACCA-3′ and a 3′ primer \textit{NF-κB} (–5.8, –6.1)R, 5′-GCTACTGACCAGCAGTCTCC-3′, was used for \textit{NF-κB} elements at –5.8 and –6.1 kb. The PCR products were subjected to direct sequencing with an ABI PRISM 3100 Genetic Analyzer. Samples from 48 patients with malaria (16 patients with noncerebral severe malaria, 16 patients with cerebral malaria, and 16 patients with mild malaria) who possessed 1 long microsatellite allele (≥15 repeats) were analyzed.

Statistical analysis. The frequencies of the genotypes with the longer microsatellite alleles (either allele of ≥15 repeats) were compared between severe malaria and patients with mild malaria using the \(x^2\) test based on a 2 × 2 table. To examine the association of the summed repeat number of both alleles with the severity of malaria, a logistic regression analysis, in which age and the summed repeat number were independent variables, was carried out using SAS software release 6.12 (SAS Institute). A Wald \(x^2\) test was used for the evaluation of odds ratios (ORs) in the logistic regression analysis.

Results

Allele frequency of the –954G→C polymorphism. The –954C allele was detected in 1 of 75 patients with malaria; the frequency of the –954C allele is estimated to be 0.0067 among Thai patients with malaria. We conclude, therefore, that this polymorphism does not significantly contribute to the severity of malaria in the studied population.

Allele frequency of the \textit{CCTTT} microsatellite repeat polymorphism. The number of microsatellite repeats ranged from 7 to 19 in the Thai population (figure 1) and did not show a bimodal distribution, as observed in the Gambian population [8]. Alleles of ≤13 repeats were found more frequently in patients with mild malaria than in patients with severe malaria, whereas alleles of ≥13 repeats (except for those of 18 repeats) were found more frequently in patients with severe malaria than in patients with mild malaria. A marked difference in the frequency was observed for alleles of ≥15 repeats. Table 1 shows the genotype frequencies of longer forms of \textit{CCTTT} microsatellite repeat (either allele of ≥15 repeats) in patients with malaria. The genotypes with longer alleles were significantly associated with severe malaria. Although no significant difference in the frequencies of the genotypes with longer alleles was detected when only 104 patients who had cerebral malaria among the 256 patients with severe malaria were examined.

Figure 1. Allele frequency of the \textit{CCTTT} microsatellite repeat polymorphism in the inducible nitric oxide synthase promoter. Data are from 179 patients with mild malaria (open bar) and 256 patients with severe malaria (closed bar) living in northwestern Thailand.
Table 1. Association of longer forms of CCTTT microsatellite repeat in the inducible nitric oxide synthase promoter with severe malaria in Thailand.

<table>
<thead>
<tr>
<th>Genotypea</th>
<th>Patients with mild malaria</th>
<th>Patients with severe malaria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 179)</td>
<td>(n = 152)</td>
</tr>
<tr>
<td>Long</td>
<td>25 (14.0)</td>
<td>44 (28.9)</td>
</tr>
<tr>
<td>Short</td>
<td>154 (86.0)</td>
<td>108 (71.1)</td>
</tr>
</tbody>
</table>

- a Long, allele of >15 repeats; short, allele of <15 repeats.
- b Patients with noncerebral severe malaria vs. patients with mild malaria (odds ratio [OR], 2.51; 95% confidence interval [CI], 1.45–4.35; χ² test, P = .0008).
- c Patients with cerebral severe malaria vs. patients with mild malaria (OR, 1.65; 95% CI, 0.88–3.11; χ² test, P = .1173).
- d Patients with severe malaria vs. patients with mild malaria (OR, 2.14; 95% CI, 1.29–3.55; χ² test, P = .0029).

NOTE. Data are no. (%) of patients.

Table 1 illustrates the association of longer forms of CCTTT microsatellite repeat in the inducible nitric oxide synthase promoter with severe malaria in Thailand. The logistic regression analysis revealed significant differences in the allele frequencies between patients with mild and severe malaria, with ORs ranging from 1.12 to 1.65. The significance of these differences suggests a functional link between iNOS transcription and NO production, implying a role of the CCTTT repeat in the pathogenesis of severe malaria.

Discussion

Our study showed a strong association of longer forms of CCTTT repeat in the iNOS promoter with severe malaria in adult Thai patients. Of particular interest was the summed repeat number, which was found to be a significant risk factor for severe malaria. The estimated ORs for severe, noncerebral severe, and cerebral malaria were ~1.1 by logistic regression analysis, implying a functional link between iNOS transcription and the number of CCTTT repeats in the iNOS promoter. Thus, we postulate that the capacity to produce NO following iNOS up-regulation is inversely correlated with the number of CCTTT repeats in Thai patients with malaria, although the level of iNOS expression in the patients has not been examined in this study. Recently, however, no correlation between the summed repeat number and measures of NO production was reported in Tanzanian children with malaria [12]. On the other hand, a luciferase reporter gene assay showed that the level of iNOS transcription was affected by the number of repeats [13]. Therefore, the relationship among NO production by iNOS, iNOS transcription level, and the number of CCTTT repeats in patients with malaria remains to be clarified. Although the −954C allele was reported to be associated with a high level of NO enzyme activity [14], the present finding of a low frequency of the −954C allele suggests that this allele is not a major risk factor for severe malaria in Thailand, compared with CCTTT microsatellite repeat.

The role of NO in patients with P. falciparum malaria has been a major object of study in recent years; however, there is still no consensus on the role of the NO level, particularly in patients with cerebral malaria. If large amounts of NO are synthesized in cerebrovascular endothelial cells, this may cause coma in patients with cerebral malaria. In this study, no significant difference in the allele frequencies was found between patients with cerebral malaria and noncerebral severe malaria. Thus, the CCTTT repeat in the iNOS promoter may not strongly affect NO production by iNOS in the brains of patients with malaria.

There may be other important factors predisposing to cerebral malaria. Excessive production of tumor necrosis factor (TNF)-α has been suggested to induce NO production that causes coma [5]. A single nucleotide substitution, −308G→A, in the TNF-α promoter was reported to be associated with cerebral malaria in Gambian children [15], although we found no association between this polymorphism and the severity of malaria in the present set of Thai patients [16]. In order to study the possible interaction between TNF-α −308G→A and iNOS (CCTTT)n, we examined the association of the combination of these polymorphisms with severe malaria, but no significant association was detected (data not shown).

Although 4 NF-κB elements in the iNOS promoter were analyzed in this study, we have not investigated the coding region of the iNOS gene. Therefore, we cannot exclude the possibility that the association of the CCTTT microsatellite polymorphism with severe malaria is caused by the linkage disequilibrium between this polymorphism and the primary associated polymorphism that is located closely to the former. It is therefore necessary to perform variation screening of the entire iNOS gene to determine the primary polymorphism involved in severe malaria. However, if the association of the CCTTT repeat with severe malaria were due to the linkage disequilibrium with another variant, such a correlation, observed in the present logistic regression analysis, would unlikely be found. Thus, a CCTTT repeat in the iNOS promoter may play a key role in the pathogenesis of severe malaria.

To our knowledge, there are only a few reports on the significant association between the iNOS promoter polymorphisms...
and the severity of malaria. Further investigations, including functional studies, are needed to understand the exact role of the iNOS promoter polymorphisms in the pathogenesis of severe malaria.

Acknowledgments

We sincerely thank the patients who participated in this study. We wish to thank 3 anonymous reviewers for thoughtful comments on the manuscript.

References