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BRIEF REPORT

Association of Endothelial Protein C Receptor (EPCR) rs867186 Gene Polymorphism With Increased Levels of Soluble EPCR and High Risk of Severe Malaria and Fatality in Beninese Children

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The endothelial protein C receptor (EPCR)–rs867186 G allele has been linked to high plasma levels of soluble EPCR (sEPCR) and controversially associated with either susceptibility or resistance to severe and cerebral malaria. In this study, quantitative enzyme-linked immunosorbent assay and sequencing were used to assess sEPCR levels and EPCR-rs867186 polymorphism in blood samples from Beninese children with different clinical presentations of malaria. Our findings show that sEPCR levels were higher at hospital admission than during convalescence and that EPCR-rs867186 G allele was associated with increased sEPCR plasma levels, malaria severity, and mortality rate ($P <$ .001, $P = .03$, and $P = .04$, respectively), suggesting a role of sEPCR in the pathogenesis of severe malaria.

Keywords. EPCR; cerebral malaria; polymorphism; rs867186.

Malaria is a major cause of death and disease in tropical and subtropical regions, where children are at high risk of severe disease, such as cerebral malaria (CM) [1]. There is evidence of the pivotal role of *Plasmodium falciparum*–infected erythrocyte cytoadhesion to endothelial protein C (PC) receptor (EPCR) in the pathogenesis of CM [2]. The EPCR protein is encoded by the PC receptor (*PROCR)* gene, which has a DNA size of about 6 kb and is located on chromosome 20 and composed of 4 exons containing several single-nucleotide polymorphisms [3]. The A-to-G substitution in the rs867186 single-nucleotide polymorphism leading to serine/glycine substitution at position 219 of the protein was found to be associated with high plasma levels of soluble EPCR (sEPCR), which increase the risks of thrombosis and result in either protection from or predisposition to severe malaria (SM) or CM [4–7]. The main function of EPCR is to maintain the integrity of cells in the brain microvasculature by binding to activated PC (APC). which has a cytoprotective function via the cleavage of protease-activated receptor 1 at Arg46 [8]. EPCR also plays a major role in modulating coagulation, as the activation of PC via the thrombin-thrombomodulin complex exerts anticoagulant activity by suppressing thrombin generation, which has proinflammatory and proapoptotic effects converse to those of APC [8].

Infected erythrocytes sequestering in the microvasculature bind to EPCR at the same site as APC does by means of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) cysteine-rich interdomain region (CIDR) variants expressed on the surface of infected erythrocytes [2]. It has been found that parasites infecting children with SM or CM express PfEMP1 variants containing CIDRα1.1 that interact with EPCR [9]. The competitive binding of PfEMP1 to EPCR leads to inflammation, edema due to hyperpermeability, and, eventually, cell death or apoptosis. When the load of *P. falciparum*–infected erythrocytes is high, the endothelial cells lining the brain microvessels are shed from the membrane-bound EPCR into the bloodstream and can bind circulating infected erythrocytes [2]. This gives rise to sEPCR, which competitively binds to PfEMP1, releasing the binding site of the unshed EPCR to interact with the appropriate ligand for proper cellular function [8]. However, high loss of cell surface EPCR contributes to increasing sEPCR release, which in turn may increase the risk of venous thrombosis and death in SM cases [5, 10, 11]. The EPCR-rs867186 G allele was shown in several studies to be associated with increased levels of sEPCR and has been found to either predispose to or protect from SM and CM [4–7]. In the context of these controversial findings, we genotyped the rs867186 loci and assessed the sEPCR plasma level in Beninese children with different clinical manifestations of malaria, including uncomplicated malaria (UM), SM, and severe CM.

METHODS

Informed Consent

Children were recruited into the study only after their parents or guardians had signed informed consent forms.

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Ethical Statement

The study protocol was reviewed and approved by the Comité National d'Ethique Pour la Recherche en Santé, Cotonou, Benin, which provided ethical clearance for the study (no. 50; 25 October 2017; IRB00006860).

Cohort Description

Study participants were part of a cohort study carried out in the South of Benin from January 2018 to July 2019, where malaria is considered to be holoendemic, with 33 infective bites per person per year [12]. The study included children aged ≤5 years with different symptoms of clinical malaria, confirmed microscopically by the presence of *P. falciparum* monoinfection in their blood smears when they presented to the hospital. Children were classified after clinical examination into 3 main groups, established according to the World Health Organization 2011 criteria: UM, severe noncerebral malaria (SNCM), and CM.

CM was defined as Blantyre coma score (BCS) <3, and this category included children with impaired consciousness or coma resulting solely from SM. The SNCM category included children who had a BCS >2 and presented with symptoms of SM, such as acute respiratory distress syndrome, pulmonary edema, acute kidney failure, SM anemia, or abnormal liver function. The UM group was defined to include children who had detected *P. falciparum* parasitemia, along with fever, headache, or myalgia, a hemoglobin level ≥5g/dL, and no damage to any vital organs. All patients had no cause of sickness other than malaria.

A 660–base pair DNA fragment spanning the EPCR-rs867186 locus was amplified by PCR from venous blood samples spotted on 3MM Whatman filter paper (Fisher Scientific). The concentration and purity of the extracted genomic DNA were determined using a NanoDrop spectrophotometer (Thermo Fisher). The following polymerase chain reaction (PCR) cycle was performed in a thermocycler (Alpha Cycler 4; PCRmax) to amplify the EPCR rs867186 gene: 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. Purified specific PCR products were sequenced in both directions using the Sanger sequencing method (Eurofins Genomics) and primers described elsewhere [7, 10]. The rs867186 alleles were identified using sequence viewer software (FinchTV, version 1.4.0; Softpedia).

Detection of sEPCR by Enzyme-Linked Immunosorbent Assay

Patients' sEPCR plasma levels were assessed on the day of hospital admission and later during convalescence (at discharge from the hospital), using a quantitative sandwich enzyme-linked immunosorbent assay method, as described by the manufacturer (Human EPCR Immunoassay kit; R&D Systems) after dilution of plasma samples to 1:20. The final sEPCR concentration for each sample (expressed in nanograms per milliliter) was calculated from the standard curve generated by quantifying a recombinant human EPCR protein. A negative control serum samples was included in the standard curve.

Statistical Analysis

Continuous and categorical variables were compared between the 3 clinical groups (CM, SNCM, and UM), using Kruskal-Wallis tests followed by Dunn post hoc tests for multiple comparison and χ^2 tests, respectively. Nonparametric Mann-Whitney tests were used to estimate differences in sEPCR levels between genotypes, and the Hardy-Weinberg equilibrium formula was used to calculate expected genotype frequencies. Differences were considered statistically significant at $P < .05$.

RESULTS

Of the 224 patients included in this study, 57 (25.45%) had UM and 167 (74.55%) had SM, including 70 (31.25%) with deep coma, as defined by a BCS <3, who belonged to the CM group, and 97 (43.30%) with other symptoms of SM and a BCS >2, who were classified in the SNCM group. Parasitemia levels did not differ significantly between the study groups. The average age in the SNCM group was lower than that in the CM and UM groups, but this difference was mainly due to chance and does not reflect any bias in recruiting of the cohort. The patients' clinical and biological features are summarized in Supplementary Table 1, and no significant associations were found between biological parameters and sEPCR.

The genotyping results were interpretable for 224 children and showed 193 (86.16%) with the AA, 22 (9.82%) with the AG, and 9 (4.02%) with the GG genotype. The frequency of

Table 1. Potential Association Between Endothelial Protein C Receptor rs867186 Gene Polymorphism and Disease Severity, Coma, or Death Resulting From Malaria Infection

Outcome	EPCR rs867186 Genotype	Children With SM, No. (%)[a]	Children With UM, No. (%)[a]	Total No.	*P* Value[b]
Disease severity	AA	139 (83.2)	54 (94.7)	193	.03[c]
	AG + GG	19 (11.4) + 9 (5.4)	3 (5.3) + 0 (0)	31	
Coma	AA	59 (84.3)	130 (86.7)	189	.46
	AG + GG	6 (8.6) + 5 (7.1)	16 (10.7) + 4 (2.6)	31	
Mortality	AA	Died: 17 (70.8)	Survived: 170 (86.7)	187	.04[c]
	AG + GG	Died: 4 (16.7) + 3 (12.5)	Survived: 20 (10.2) + 6 (3.1)	33	

Abbreviations: EPCR, endothelial protein C receptor; SM, severe malaria; UM, uncomplicated malaria.

[a]Children with SM (which included both cerebral and severe noncerebral malaria) had a Blantyre coma score (BCS) <3; those with UM, a BCS >2.

[b]*P* values were determined using the χ^2 method.

[c]Significant at $P < .05$.

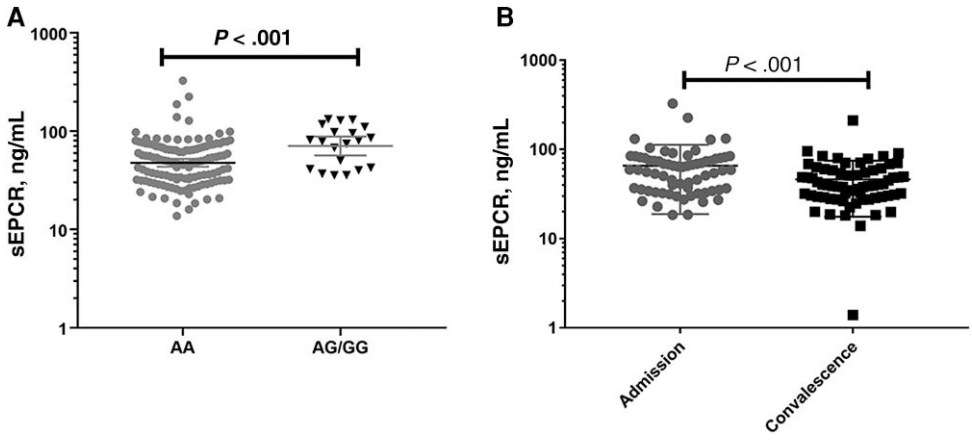

Figure 1. Plasma levels of soluble endothelial protein C receptor (sEPCR) according to patients' EPCR-rs867186 genotypes, on the day of hospital admission and during convalescence. *A,* Plasma sEPCR levels in patients with wild-type homozygotes AA (*dots*; n = 126), and in AG heterozygotes (n = 14) and GG (n = 6) homozygote mutants (*triangles*) at the EPCR rs867186 locus at hospital admission. The difference between AA and AG-GG carriers was significant ($P < .001$; Mann-Whitney test). *B,* sEPCR levels in patients at hospital admission (*dots*; n = 68) and during convalescence (*squares*; n = 68); this comparison also showed a significant difference ($P < .001$; Wilcoxon matched-pairs signed rank test).

the major allele A was 0.91, and that of the minor or mutant allele G was 0.09 (Supplementary Table 2). The distributions of genotypes according to disease severity, coma, and mortality rate are given in Table 1. We observed significantly more AG and GG genotypes in patients with SM (comprising CM and SNCM groups) than in those with UM ($P = .03$) and more in fatal cases than in survivors ($P = .04$). However, this difference was not significant when comparing BCS categories (patients with BCS <3 vs BCS >2), probably owing to the low number of individuals with the mutant allele. Among the 146 children in whom we assessed plasma sEPCR levels, a majority (n = 126 [86.3%]) had the dominant AA genotype, and these patients had significantly lower sEPCR levels than those with the AG (n = 14 [9.6%]) or GG (n = 6 [4.1%]) genotype ($P < .001$) (Figure 1*A*). Regardless of EPCR-rs867186 genotype, plasma sEPCR levels decreased significantly during convalescence compared with the day of hospital admission in the 68 children tested on both occasions ($P < .001$) (Figure 1*B*).

DISCUSSION

The controversial findings about the association between the EPCR rs867186 G allele and protection from or susceptibility to SM aroused our interest in investigating the potential relationship between this allele and malaria severity in another malaria setting. This is all the more important given the potential association between plasma sEPCR levels and the severity of malaria and the risk of death as well as the plausible relationship between high sEPCR levels and the presence of the G allele [5, 10].

Our results clearly indicate that the mutant EPCR rs867186 G allele is associated with higher plasma sEPCR levels than the wild-type A allele ($P < .001$), as shown in Figure 1*A*, in agreement with other findings [4, 5, 10]. Interestingly, we found that the AG and GG genotypes tend to be more frequent in children with SNCM or CM than in those with UM ($P = .03$), and we also found a significant difference between children who died of malaria and those who survived, ($P = .04$). These results suggest an association of AG-GG genotypes with SM, which may result from the implication of sEPCR in the modulation of PC and APC functions promoting thrombosis [13].

These results agree with those of other investigations showing the association of high levels of sEPCR with malaria severity and mortality rate and the susceptibility of rs867186 G allele carriers to SM and increased risk of death, findings consistent with the association of this allele with high sEPCR levels [6, 10]. However, our results were contrary to those of other studies showing a protective effect of the rs867186 G allele for SM in Ugandan and Thai populations [4, 7]. In one of these studies sEPCR was not assessed; in the second, the sEPCR levels were higher in children with UM than in those with SM, but the difference was significant only in the heterozygote group, in strong contrast to our group's earlier findings (which included the present data) showing significantly higher sEPCR levels in the SNCM and CM groups than in the UM group [11]. In the present study, differences between the SNCM-CM and the UM groups, in term of genotypes and sEPCR levels, were not detected, but this may be owing to the limited number of individuals for whom both data sets were available.

It should also be noted, however, that of the 3 statistical models used in the published studies, only the "recessive model" showed a significant association of the rs867186 G allele with protection from CM [4]. Therefore, the protective effect of the rs867186 G allele for SM remains to be explained in the context of high plasma sEPCR levels conferred by this mutation [4]

and the described association between increased sEPCR levels and the risks of SM and death [8, 10] as well as thrombosis.

In contrast to published results [13], we found significantly higher sEPCR plasma levels at hospital admission than during convalescence ($P < .001$) and in severe compared with mild cases, strengthening the association of high sEPCR levels with malaria pathology. This might be the result of a protective mechanism counteracting the binding of infected erythrocytes to cellular EPCR at the microvasculature level, an eventuality consistent with the inhibition of PfEMP-1 DC8–binding-parasite to endothelial cells observed in vitro [14]. If unambiguously confirmed in vivo, this hypothesis supports the development of adjunct therapies based on specific variants of sEPCR, such as E86A-sEPCR, which binds to CIDRα1.1 without interacting with PC, thus allowing the cytoprotective effects of APC on endothelial cells [14]. The discrepancies in findings between different studies might also result from differences in patients' clinical state or ethnicity or differences in parasite populations.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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