A meta-analysis of the endothelial protein C receptor rs867186 genotype in malaria

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Abstract

BACKGROUND : During *P. falciparum* infection, the binding of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) to endothelial cells (EC) results in the sequestration of pRBC. Several receptors located on the endothelial cells, including intercellular adhesion molecule 1 (ICAM-1), CD36, and endothelial protein C receptor (EPCR), contribute to PfEMP1 adhesion to the microvasculature. PfEMP1, expressed on the surface of parasitized red blood cells (pRBC), is composed of cysteine-rich interdomain regions (CIDR) and Duffy binding-like (DBL) domains. CIDRa1 competitively binds to EPCR with activated protein C (APC) and impairs cytoprotective and anticoagulant effects by APC, which plays important roles in severe malaria (SM) pathogenesis such as cerebral malaria (CM) and severe malaria anemia (SMA). The strategy to inhibit EPCR binding to pRBC while concomitantly strengthen its binding to APC may be crucial in restoring disrupted protein C (PC) system's function. The purpose of this study is to evaluate the association between malaria severity and the EPCR genotypes as well as with soluble EPCR (sEPCR), and the study also addresses the physiological relevance of EPCR genetic polymorphism. **RESULTS :** In this study, we conducted a meta-analysis on the eligible studies by comparing the frequency of EPCR rs867186-GG versus rs867186-GA and -AA genotype in SM, mild malaria (MM) or uncomplicated malaria (UM) patients and healthy individuals from Thailand, Uganda, Benin, Tanzania, and Ghana. We also determined the relationship between rs867186 genotype and sEPCR levels. Our results showed that the genotype rs867186-GG is higher in MM/UM than in SM patients. SM patients carrying the rs867186-GG genotype have higher plasma soluble EPCR (sEPCR) levels than in rs867186-AG and rs867186-AA carriers. MM/UM patients carrying the rs867186-AG genotype have significantly higher level of sEPCR compared to those carrying rs867186-AA. Similarly, the rs867186-GG is associated with high sEPCR level in healthy individuals. **CONCLUSIONS :** This meta-analysis demonstrates that pRBCs and EPCR interactions are associated with malaria severity, and treatments that block their binding via PfEMP1 CIDRa1 could be a potential therapy for SM.

Introduction

Severe malaria (SM) is defined as cerebral malaria (CM), severe malaria anemia (SMA), or malaria patients with high levels of parasitemia, hypoglycemia, and increased creatinine, which demonstrates high mortality in patients with *P. falciparum* infections [1, 2]. The pathological feature of SM is the sequestration of pRBC to host microvasculature beds in a variety of organs and tissues due to their binding to endothelial cells (known as cytoadherence) via the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) [3-9]. PfEMP1 protein is encoded by approximate 60 *var* genes with each parasite expressing only one *var* gene at a time [10]. The extracellular part of PfEMP1 is composed of cysteine-rich interdomain regions (CIDR) and Duffy binding-like (DBL) domains [11-13]. DBL-CIDR in tandem within N-terminus is referred to as the semi-conserved protein head structure and is the most conserved extracellular region [14]. CIDRa1 domains of PfEMP1 bind endothelial protein C receptor (EPCR) [11, 15], while CIDRa2-6 domains of PfEMP1 bind to CD36 [11, 15], and DBL β 5 domains bind to intercellular adhesion molecule 1 (ICAM-1) [13, 15]. The parasite sequestration, which is associated with disease severity, is determined by the binding of PfEMP1 to host endothelial cell receptors mentioned above [16-18]. pRBC binding to CD36 on endothelial cells (or platelets

and monocytes) usually correlates with uncomplicated or mild malaria (UM/MM), whereas pRBC adhesion to ICAM-1 is a characteristic of SM [16-18]. Recently, Turner et al. discovered that EPCR interactions with PfEMP1 domain cassette 8 (DC8) and DC13 (both of which contain CIDRa1 domain) correlated with SM [19]. Following their study, several research groups further showed that the binding of CIDRa1 to EPCR competitively inhibits activated protein C (APC)'s binding to EPCR, inducing pro-apoptotic and pro-inflammatory process and concomitantly inhibiting cytoprotection, anticoagulation, and barrier functions in hosts [20-22]; ultimately, CIDE α 1's competitive inhibition leads to SM, including CM and SMA [23]. By using qRT-PCR to test different subsets of PfEMP1 *var* genes on CM samples from Ugandan children, Shabani et al. demonstrated that the EPCR-binding PfEMP1 transcript levels are important for the development of SM [12]. They found that children with combined CM and SMA expressed the highest number of EPCR-binding PfEMP1 transcripts, noting that as disease severity decreases, the number of EPCR-binding PfEMP1 transcripts decreases as well [12]. Their findings of the association between the increased EPCR-binding PfEMP1 (*var* CIDRa1 domain) transcript and the severity of malaria indicate that EPCR-binding PfEMP1 transcript could be a novel therapeutic target for SM. The abundance of the transcripts increased with malaria severity; however, each subtype transcript did not vary remarkably among patients, which suggests that all EPCR-binding CIDRa1 subtypes are required to be targeted to deplete *P. falciparum* sequestration in host blood circulation, in particular for sequestration in brain blood circulation [11]. Several lines of evidence support a correlation between the plasma EPCR (soluble EPCR, sEPCR) levels and the genetic polymorphisms of the *EPCR* gene, particularly the EPCR rs867186-A/G genotype [1, 2, 24-26]. sEPCR can competitively bind to the CIDRa1 domain and therefore displaces EPCR binding sites for APC, which restores cellular EPCR function. Due to a lack of systemic analysis of EPCR genotypes, sEPCR levels, and their relationship with malaria disease severity, in the present study, we performed a meta-analysis based on related literatures to determine the clinical significance of the EPCR gene in SM pathogenesis.

Methods

Search strategy

Pubmed, Medline, Web of Science, Scopus and Embase were searched until April 2019 using the following terms: "endothelial protein C receptor," "EPCR," "malaria," "severe malaria/complicated malaria/uncomplicated malaria," "cerebral malaria," "severe malaria anemia," "polymorphism," and "clinical studies." Articles screened were obtained by titles first and then by article abstracts. After excluding non-relevant publications, the remaining papers were evaluated in the full-text version based on in- and exclusion criteria. All searched data were retrieved, and the language of publication was restricted to English.

Selection criteria

In this meta-analysis, we collected all eligible publications regarding the correlation between EPCR and clinical outcomes and clinicopathological features in malaria patients. Studies identified met the following the requirement of inclusion: (1) rs867186-A/G genotype was analyzed and evaluated using DNA from peripheral blood mononuclear cells; (2) articles that studied the correlation between rs867186-AG genotype

and malaria severity; (3) articles that studied SM using similar criteria, which was defined as the presence of one of the following signs: high *P. falciparum* parasitemia ($>100,000$ parasites/ μ l), hypoglycaemia (glucose level <22 nmol/l), severe anemia (hematocrit $<20\%$ or hemoglobin <7 g/dl), increased serum creatinine (>3 mg/dl), and unrousable coma (or a Blantyre Coma Score below 3) caused by malaria infection regardless of other signs (cerebral malaria); and (4) studies with hazard ratio (HR) and 95% confidence interval (CI) that can be estimated. The exclusion criteria includes: (1) letters, case reports, reviews, editorials, conference abstracts, and expert opinion; (2) studies without information on the genotype of the AA and AG alleles of rs867186 (rs867186-AG genotype); and (3) all in vitro/ex vivo studies including cell culture and animal studies.

Data extraction

Two investigators independently extracted data from eligible studies. Disagreements were resolved through full discussion until consensus was reached. The following data was identified and recorded for each study: year of publication, the first author's name, number of cases, sample source, rs867186-AG genotype, and clinicopathological parameters. Data for study characteristics were collected and summarized, and the heterogeneity of each study was evaluated as well.

Statistical analysis

The data analysis was performed using the software Review Manager 5.3 (Cochrane Collaboration, Oxford, UK) and Stata 12.0 (Stata Corporation, TX, USA). Comparisons of dichotomous measures were conducted by pooled odds ratios (ORs). Heterogeneity was determined by a chi-square test with significance set at $P < 0.10$; the total variation among studies was determined by I square. The I square value is an estimate of variance due to between-study heterogeneity rather than chance (the Cochran Q statistics). P-value of <0.05 was considered to be statistically significant. If there were heterogeneity among studies (when I square exceeded 50%), we used a random-effect model to pool the ORs; otherwise, a fixed-effect model was selected.

Results

1. Identification of relevant studies and study characteristics

Twenty-eight articles were identified, and twenty-three of those were excluded due to non-original articles (review), laboratory studies, and studies irrelevant to the current analysis. Finally, there were five studies that met the selection criteria and were included in the final meta-analysis (Fig. 1). Five studies, published from 2014 to 2016, were eligible for meta-analysis [1, 2, 24-26]. A total of 2995 SM, 487 uncomplicated malaria, and 2105 healthy controls from Thailand, Uganda, Benin, Tanzania, and Ghana were enrolled. The characteristics are listed in Table 1. The definition criteria for the five manuscripts' SM are presented in Table 2.

2. rs867186 A>G and the risk of malaria

We compared the genotype frequencies of GG (rs867186-GG) versus AA (rs867186-AA) alleles of rs867186 in malaria patients and healthy individuals. We observed that the prevalence of rs867186-GG is not significantly higher in healthy controls than in malaria patients. The pooled OR from 3 studies, including 2532 patients with malaria and 2105 healthy individuals, is shown in Fig. 2 (OR=0.92, 95% CI=0.74-1.15, P=0.49).

3. Association of rs867186-G variant with higher plasma soluble EPCR (sEPCR) levels

Two studies were eligible to analyze the relationship among PROCR gene, rs867186-G (GG or AG) variant, and plasma soluble EPCR levels. In the Beninese studies conducted by Moussiliou et al. [24], no patient had the homozygous recessive genotype rs867186-GG. At convalescence, the overall analysis of genotype showed higher plasma sEPCR levels among CM and SM patients carrying the rs867186-AG genotype compared with those carrying rs867186-AA; data is shown in Table 3 (297ng/ml vs. 158ng/ml, respectively; P=0.03). There is an upward trend of plasma EPCR levels among CM and SM patients carrying the rs867186-AG genotype compared with those carrying rs867186-AA (P=0.17) at admission, but they are not significantly different [24]. The studies from Shabani [26] showed that CM and SMA patients carrying rs867186-GG or rs867186-AG genotype have higher sEPCR levels than rs867186-AA carriers (194ng/ml and 131ng/ml vs. 84.5ng/ml, P=0.007 and P<0.001, respectively, Table 3). In addition, a clear difference was observed with higher plasma sEPCR levels among uncomplicated malaria patients carrying the rs867186-AG genotype compared to those carrying rs867186-AA (161ng/ml vs. 86.5ng/ml, P<0.001, Table 3). Similarly, the rs867186-GG was associated with higher sEPCR levels in healthy individuals (350ng/ml vs. 241ng/ml, P<0.006, Table 3). Our results show the association between the rs867186-G variant and higher circulating sEPCR levels both in SM patients, including CM and SMA, in high *P. falciparum* parasitemia burden (>250,000 parasites/μl) [24, 26], and in UM/MM and healthy individuals.

4. Association of rs867186-GG genotype with the protection of SM

We analyzed genotype frequencies of the GG alleles (rs867186-GG) versus AA and AG alleles (rs867186-AA+AG) of rs867186 in SM and uncomplicated/mild malaria (UM/MM) patients. We observed that the prevalence of rs867186-GG is higher in mild malaria than in SM patients. The pooled OR from 2 studies, including 892 patients with SM and 437 mild malaria is shown in Fig. 3 (OR=0.36, 95% CI=0.14-0.91, P=0.03). The possible reason that the rs867186 genotypes distinguish between mild malaria and SM but not healthy controls and malaria patients (Fig. 3) is due to healthy controls lacking the pressure exerted by parasites of the genus *Plasmodium*, which causes malaria infection.

5. Sensitivity analyses and publication bias

A sensitivity analysis is an important part of a meta-analysis as it aims to determine the robustness of the observed outcomes. A sensitivity analysis was performed to assess the results' stability. We perform the sensitivity analysis by excluding one study at a time to see whether or not the results remained consistent. The results showed that the pooled ORs are not significantly changed, indicating the stability of analytic results. The funnel plots are largely symmetric, indicating that there are no publication biases in the meta-analysis of the role of rs867186-GG genotype in SM (Fig. 4).

Discussion

The cell surface protein of EPCR was originally cloned in 1994 [27]. Multiple ligands of EPCR have been found, including PfEMP1, protein C (PC)/activated protein C (APC), factor VIIa, tissue factor, and a specific variant of the T-cell receptor. Under physiological conditions, Protein C is activated by the thrombin-thrombomodulin complex, and activated protein C (APC) then cleaves the protease-activated receptor (PAR1) at Arg46, which triggers an anti-apoptotic and anti-inflammatory reaction to inhibit thrombin production and stabilizes endothelial barrier function [21, 28, 29]. In SM, PfEMP1 binding to EPCR, which blocks APC to EPCR. Consequently, thrombin signaling in the absence of modification by APC/EPCR has strong pro-inflammatory effects, upregulation of NF κ B with increased tumor necrosis factor (TNF) and interleukin (IL)-6 production, reduction of Angiopoietin 1 (Ang1) production, and contributes to apoptosis, inflammation, loss of endothelial barrier integrity, and leak [11, 13, 22].

Each *Plasmodium falciparum* genome holds about 60 PfEMP1 encoding var genes. DC13-containing PfEMP1 variants can bind to both the EPCR and ICAM-1 [22]. Both ICAM-1 [30-32] and EPCR [9, 33-36] are involved in the pathogenesis of CM, for instance, ICAM-1: PfEMP1 binding and resetting have been identified as one of the virulence factors [37], while EPCR-binding parasites dominate host infections with limited anti-malaria immunity [9, 33-36]. sEPCR is shed from cellular EPCR on the surface of endothelial cells, and they share the same binding activity; therefore, the addition of recombinant sEPCR inhibits the binding among PfEMP1-DC13 and -DC8 variants and cellular EPCR on endothelial cells [19], which consequently increases the risk of venous thrombosis [19, 38]. Thus, it seems individual having EPCR genotype is being protected against SM at the expense of a higher risk for thrombotic disease [19, 38].

To assess the effect of rs867186-GG polymorphism for the risk of SM, we analyzed genotyped patients with mild malaria and SM on rs867186-GG of previous publications. We found that the rs867186-GG genotype appears significantly more frequent in patients with mild malaria than those with SM ($P=0.03$ in Fig. 3). However, when we compared genotypes rs867186-GG versus rs867186-AA in malaria patients and healthy individuals, the frequency of rs867186-GG in malaria patients and healthy controls is very similar ($p=0.90$ in Fig. 2). This was not caused by studies bias, as the heterogeneity is calculated as $I^2=0$ (Fig. 2), and this implies that we can take a fixed-effect model to pool the ORs rather than a random effect model. The genotype difference of the GG and AA alleles of rs867186 is likely due to the [pressure](#) exerted by parasites of the genus *Plasmodium* that cause malaria. Thus, comparing healthy controls that lack these pressure with any disease status is unnecessary. This is a possible reason that these genotypes distinguish between mild and SM but not between healthy controls and malaria patients. The studies from Hansson et al. [1] and Schuldt et al. [25] reported that the genotype rs867186-GG does not have a protective role in malaria patients compared to healthy controls. However, they did not compare the difference in rs867186-GG genotype between SM and MM.

The metalloprotease cleaves the surface cellular EPCR, which releases a soluble EPCR (sEPCR) that circulates in the plasma [19]. The relationship between the polymorphisms in EPCR and plasma sEPCR levels has been conducted in patients with thrombosis to evaluate the risk of venous thrombosis by Medina et al. [39]. Their data indicate that individuals carrying some specific genotypes have high sEPCR and APC

levels, thus having a lower risk of venous thromboembolism [39]. However, no systemic analysis has been made in malaria patients regarding the EPCR genotype and plasma EPCR levels. When we examined the relationship between rs867186 polymorphisms and plasma sEPCR evaluating risk of SM, we found that the carriers of the rs867186-GG genotype have significantly higher sEPCR levels than those with the AG and AA genotypes in SM; carriers of the rs867186-AG genotype have significantly higher sEPCR levels than those with AA genotype in uncomplicated malaria, and carriers of the rs867186-GG genotype have significantly higher sEPCR levels than those with AG genotype in healthy individuals (Table 3). These results support that the rs867186 GG genotype is associated with elevated sEPCR levels in SM (Table 3). The loss of endothelial protein C receptor link coagulation and inflammation to parasite sequestration in CM [26, 40], and rs867186-GG is associated with increased soluble EPCR and can potentially mediate protection against SM.

Two studies involving adult malaria patients [2, 12] revealed that the EPCR rs867186-G allele could mediate protection against SM, while the other three studies involving child malaria patients showed that EPCR gene variants are not associated with SM [1, 25] or increased mortality among children with CM [24]. Although they utilized similar criteria for SM, adult and child malaria may have different pathophysiology. Once more studies in this field are available in the future, the EPCR polymorphism study should be conducted on adult and child malaria separately.

Conclusions

This meta-analysis summarizes previous observations and indicates the EPCR rs867186-GG genotypes are associated with increased soluble EPCR and may have an important role in SM. pRBCs bind to EPCR on endothelial cells and block APC's access to EPCR, inducing pro-apoptotic, pro-inflammatory, and loss of local vascular barrier integrity. Targeting EPCR-PfEMP1 binding can potentially reverse *P. falciparum* sequestration in host blood circulation, and in particular for sequestration in brain blood circulation, which could be an effective intervention to prevent SM. In the future, more studies are needed to assess the adhesive abilities of PfEMP1 expressed in isolation of pRBC from SM, CM patients, and even pregnant women with malaria. Furthermore, the mechanism of EPCR genotype protecting individuals against SM leads to an increased risk of thrombotic disease is also worthy of exploration.

Although we only found five publications with a total of 2995 SM and 487 uncomplicated malaria, performing a meta-analysis is justified since we are working with a fixed-effect. A summary based on two or more studies yields a more precise estimate of the true effect than either study alone.

Abbreviations

Ang1	Angiopoietin 1
APC	Activated protein C
CIDR	Cysteine-rich interdomain regions
CM	Cerebral malaria

DBL	Duffy binding-like
DC8	Domain cassette 8
EC	Endothelial cells
EPCR	Endothelial protein C receptor
ICAM-1	Intercellular adhesion molecule 1
MM	Mild malaria
ORs	Odds ratios
PC	Protein C
PfEMP1	<i>P. falciparum</i> erythrocyte membrane protein 1
pRBC	Parasitized red blood cells
sEPCR	Soluble endothelial protein C receptor
SM	Severe malaria
SMA	Ssevere malaria anemia
UM	Uncomplicated malaria

Declarations

Authors' contribution

LY and ML contributed substantially to the study design, data acquisition, analysis, and interpretation of data. RX and SG contributed substantially to the acquisition, analysis, interpretation of data, and performed the statistical analysis. LY and ML have been involved in the drafting process and critical revision of the article for important intellectual content. The corresponding authors had full access to all data and the final responsibility for the decision to submit the article for publication. All authors read and approved the final manuscript.

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There is no founding in this study.

Availability of data and materials

All the data required is included in the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interest

The authors have no financial relationships and conflicts of interest in the manuscript.

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Supplementary material

PRISMA Checklist

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Tables

Table 1. Basic characteristics of the included studies

Study	Year	Population	SM No. of cases			MM No. of cases			HC No. of cases			SNP	Conclusion Reported
			GG n	GA n	AA n	GG n	GA n	AA n	GG n	GA n	AA n		
Naka	2014	Thailand	5	94	242	16	88	262				rs867186	Protective
Schuldt	2014	Ghana	108		1797				110		1756	rs867186	No association
Hansson	2015	Tanzania	8	68					7	60		rs867186	No association
Moussiliou	2015	Benin		19	103		9	41				rs867186	High mortality
Shabani	2016	Uganda	3	102	446	0	14	57	7	31	134	rs867186	Protective

Table 2. Definition criteria for severe malaria

Study	Severe malaria				
Naka 2014	High <i>Pf</i>	SMA	hypoglycemia	High serum creatinine	CM
Schuldt 2014	High <i>Pf</i>	SMA			CM
Hansson 2015	High <i>Pf</i>	SMA			CM
Moussiliou 2015	High <i>Pf</i>	SMA			CM
Shabani 2016		SMA			CM

High *Pf*: >100,000 parasites/ μ l

SMA: severe anemia: hematocrit (HCT) <20%, hemoglobin (Hb) <7g/dl

CM: cerebral malaria

High serum creatinine: serum creatinine >3mg/dl

Hypoglycaemia: glucose <22 nmol/l

Table 3: rs867186-G variant is associated with higher sEPCR levels in severe malaria

Study	Stages	Genotype (n=number)	Plasma levels of sEPCR		
			Median (ng/ml)	Interquartile range (IQR)	P value
Moussiliou 2015	#CM+###SM	AA	158	131-211	P=0.03
	Convalescence	AG	297	174-383	
Shabani 2016	CM+ ##SMA	AA(n=390)	84.5	65.7-104	*P<0.001 **P=0.007 ***P=0.71
		AG (n=91)	131	107-170	
		GG(n=3)	194	104-211	
	§UM	AA (n=25)	86.5	75.4-113	P<0.001
		AG (n=13)	161	142-164	
	§§Healthy CC	AA (n=79)	98.4	87.8-121	P<0.006
		AG (n=25)	241	203-288	
		GG (n=6)	350	319-380	

*AG compared with AA, ** GG compared with AA, *** GG compared with AG

#CM (cerebral malaria), ##SMA (severe malaria anemia), ###SM: severe malaria: >250,000 parasites/ μ l, or SMA

- UM: uncomplicated malaria, §§Healthy CC: healthy community controls

Figures

Figure 1

Flow chart of the study. Among twenty-eight publications identified by the search method, twenty-three of those were excluded due to exclusion criteria of laboratory studies, non-original articles, lack of matched controls, or studies irrelevant to the current analysis. Five studies were included in this meta-analysis.

Figure 2

Forest plot for the risk of malaria and EPCR polymorphism in the genotype of rs867186 GG vs. AA. The frequency of rs867186-GG versus rs867186-AA in malaria patients and healthy individuals were compared. The prevalence of rs867186-GG is not higher in healthy controls than in SM patients. The pooled OR from 3 studies, including 2532 patients with malaria and 2105 healthy individuals, is shown (OR=0.92 95% CI=0.74-1.16, P=0.49).

Figure 3

Forest plot for the protection of SM and EPCR polymorphism in the genotype of rs867186 GG vs. AG and AA. The frequency of rs867186-GG versus rs867186-AA and AG was analyzed in SM and

uncomplicated/mild malaria patients. The frequency of rs867186-GG is higher in mild malaria than in SM patients. The pooled OR from 2 studies, including 892 patients with SM and 437 mild malaria, is shown (OR=0.36, 95% CI=0.14-0.91, P=0.03).

Figure 4

Funnel plot of publication bias in the meta-analysis of rs867186 genotype. The funnel plots are largely symmetrical, suggesting there are no publication biases in the meta-analysis of the role of rs867186-GG genotype in SM.

Supplementary Files

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