Promoter Polymorphisms in the ATP Binding Cassette Transporter Gene Influence Production of Cell-Derived Microparticles and Are Highly Associated with Susceptibility to Severe Malaria in Humans

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Microparticle (MP) efflux is known to be mediated by the ABCA1 protein, and the plasma level of these cell-derived MPs is elevated considerably during human malarial infection. Therefore, two polymorphisms at positions –477 and –320 in the promoter of the ABCA1 gene were genotyped and tested for association with the plasma MP level in four groups of malaria patients segregated according to the clinical severity, i.e., cerebral malaria (CM), multiorgan dysfunction (MOD), noncerebral severe malaria, and uncomplicated malaria (UM). The TruCount tube-based flow cytometric method was used for the exact quantification of different cell-derived MPs in patients. Polymorphisms in the ABCA1 gene promoter were analyzed by use of the PCR/two-primer-pair method, followed by restriction fragment length polymorphism, in 428 malaria patients. The level of circulating plasma MPs was significantly higher in febrile patients with *Plasmodium falciparum* infection, especially in CM patients compared to healthy individuals. The homozygous wild-type –477 and –320 genotype was observed to be significantly higher in patients with severe malaria. These patients also showed marked increases in the plasma MP numbers compared to UM patients. We report here for the first time an association of ABCA1 promoter polymorphisms with susceptibility to severe malaria, especially to CM and MOD, indicating the protective effect of the mutant variant of the polymorphism. We hypothesize that the –477T and –320G polymorphisms affect the downregulation of MP efflux and may be a predictor of organ complication during *P. falciparum* malarial infections.

A state of dysregulation of the haemostatic system by systemic endothelial activation, endothelial damage (1), low blood cell counts, and the generation of microparticles (MPs) is associated with clinical *Plasmodium falciparum* malaria infection (2). The disease has a high mortality (3) due to the intensity of the coma (4) and the rapidity of its progress. The “immunological theory” explaining the severe malaria (SM) excessive response is accredited to the sequestration of activated blood cells in capillary beds by the adhesion to vascular endothelium of the central nervous system, predominantly in the deep tissues consequent to the host inflammatory response (5). This inflammatory process results in the activation of cells and the production of potent factors known as MPs (6, 7). MPs are cell-derived phospholipid vesicles in the bloodstream shed by virtually all cell types (8) that are activated by a variety of stimuli (7, 9) and circulate in healthy humans, where their development is a tightly regulated haemostatic process (10), and their numbers are increased in various pathological conditions such as thrombotic (11), cardiovascular (12), and infectious diseases (13, 14). In particular, their association in cerebral dysfunction during SM has been studied (15).

ATP binding cassette (ABCA1) transporter is a key controller of cellular lipid handling and was initially identified as a phosphatidylserine (PS) flippase that translocates PS from the inner leaflet to the outer leaflet of the plasma membrane (16). At the cellular level, ABCA1 plays a key role in cholesterol efflux and transfer from peripheral cells to lipid-poor Apo A-I, the first step in high-density lipoprotein (HDL) particle formation (17). In addition to the efflux of cholesterol to Apo A-I, a recent study shows that ABCA1-expressing cells also release substantial amounts of cholesterol in the form of MPs (18). Dysfunctional mutations of ABCA1 gene in humans result in Tangier disease (19), a clinical

disorder characterized by the near absence of circulating HDLs and high incidences of cardiovascular disease (20).

The ABCA1 transporter is known to participate in infectious and/or thrombotic disorders involving MP formation, since homozygous ABCA1 gene deletions confer complete resistance against cerebral malaria (CM) in mice (6). This was seen in a recent observation of the absence of the ABCA1 gene in a mouse model with very low levels of circulating MPs and complete resistance to CM, suggesting a major role for the ABCA1 protein in regulating the plasma MP in human malaria as well. An earlier study conducted in coronary atherosclerosis (21) identified –477C/T and –320G/C polymorphisms in the promoter of the ABCA1 gene that were associated with a defect in reverse cholesterol transport, thereby causing increased cholesterol accumulation in the vessel wall and increased atherosclerotic burden. That study tried to determine whether these polymorphisms in the ABCA1 gene caused any alteration in the amount of MP efflux from the cells in human malaria patients and to assess the functional relevance of these polymorphisms, if any, in human malaria.

In the present study, we associate the polymorphisms in the ABCA1 gene promoter with the clinical status of the malaria patients and address the genetic basis of production of MPs during *P.*

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falciparum infection in patients with SM compared to patients with uncomplicated malaria (UM). The levels of circulating cell-derived MPs, namely, erythrocyte-derived, platelet-derived, and endothelial-cell-derived MPs, were calculated to precisely in different malaria patients segregated according to the severity of the disease and, in this manner, we assessed the importance of the polymorphisms in the ABCA1 promoter in affecting plasma levels of the MP phenotype and in serving as genetic risk factors affecting the clinical severity of *P. falciparum* malaria in the general population.

MATERIALS AND METHODS

Study area and selection of study subjects. The study was conducted from December 2008 to September 2010 in SCB Medical College and Hospital, Cuttak, Odisha, India. The state is considered to be hyperendemic for malaria, and the transmission is perennial, with a seasonal peak (July to October). All four species of human malaria are found in Orissa, but >85% of all cases of clinical malaria are due to *P. falciparum* (22). Clinically suspected malaria patients were screened for *P. falciparum* infection using both microscopy (23) and species-specific PCR diagnosis (24). The clinical categorization of malaria was classified according to the definitions and associated characters published by the World Health Organization (WHO) (25). Uncomplicated malaria (UM) was defined as patients with an axillary temperature of >37.5°C, symptoms of headache, fever, and myalgia, evidence of *P. falciparum* infection in the blood, no schizontemia, no intake of antimalarial drugs within the preceding week, and no history of hospitalization (to exclude those who already had a severe malaria [SM] attack). SM was categorized into three distinct clinical groups, namely, cerebral malaria (CM), noncerebral severe malaria (NCSM), and multiorgan dysfunction (MOD). CM was further defined as patients with an altered sensorium and a GCS (Glasgow coma scale) of ≤10. NCSM patients had one of the several manifestations of SM without cerebral involvement, namely, severe anemia (hemoglobin, <5 g/dl), acute renal failure (serum creatinine, >3 mg/dl), jaundice (serum bilirubin, >3 mg/dl), acute respiratory distress syndrome (ratio of the partial pressure of arterial O₂ to the fraction of inspired O₂ [Carrico index]: PaO₂/FIO₂, <200), hemoglobinuria (dark red or black urine positive for hemoglobin), and shock (systolic blood pressure of <80 mm Hg). MOD was diagnosed based on the involvement of two or more organs such as the central nervous system (Glasgow coma scale [GCS], ≤10), respiratory (PaO₂/FIO₂, <200), renal failure (serum creatinine, >3 mg/dl), and hepatic dysfunction (alanine aminotransferase/aspartate aminotransferase [ALT/AST] more than three times of normal, prolonged prothrombin time, and albuminemia). After informed consent was obtained from all enrolled patients found to be positive for *P. falciparum* on the day of reporting (day 0) before the administration of any antimalarial drugs, as well as healthy controls (HC) of the same ethnicity and hailing from a similar geographical background with no reported history of clinical malaria in the last 5 years or suffering from any other infections, venous blood was collected in 0.129 mol of trisodium citrate/liter (for MP analysis) as the anticoagulant at a ratio of 9:1 and EDTA (for malaria diagnosis and ABCA1 gene analysis) where the risk of exposure to malaria was similar for both HC and patients. The exclusion criteria included (i) confirmed diagnosis of coinfection with other *Plasmodium* species, (ii) the presence of symptoms of UM or SM with other acute infections, including intestinal geohelminthic infections (26), (iii) chronic diseases such as tuberculosis, leprosy, and malnutrition, (iv) genetic disorders, such as hemoglobinopathies and G6PD deficiency (27), and (v) all pregnant women, smokers, and patients with coronary artery diseases and diabetes mellitus. Each patient was treated according to the local guidelines and care was provided until discharge from the hospital, and patients with mild malaria were followed up until they were free from clinical symptoms and parasitemia. The study and its protocol were approved by the Ethical Committee of the Regional Medical Research Centre, Bhubaneswar, Orissa, India.

Flow cytometric detection of MPs. Platelet-free plasma (PFP) was prepared by sequential centrifugation of the citrate anticoagulated blood collected stored at −80°C until further analysis. MPs were isolated from PFP after a two-step sequential ultracentrifugation procedure as described previously (28, 29). The MP suspension was prepared by adding 250 μl of the PFP to 10 ml of freshly prepared washing buffer (10 mM HEPES, 104 mM NaCl, 4.5 mM KCl, 1% bovine serum albumin, 0.1% sodium azide, 2.5 mM CaCl₂; pH 7.4 [the buffer was filtered through a 0.2-μm-pore-size filter]), maintaining a ratio of 1:400. The mixture was centrifuged at 100,000 × g for 70 min at 20°C in an ultracentrifuge. The supernatant was carefully aspirated out, leaving 1 ml of residue. The pelleted MPs were resuspended again in the washing buffer (maintaining a ratio of 1:100) by gentle vortexing and centrifuged under the same conditions (100,000 × g for 70 min at 20°C). Finally, the top 90% of supernatant was removed, leaving an MP-enriched fraction. The pelleted MPs were resuspended in the remaining wash buffer by gentle pipetting. The MP suspension was aliquoted (90 μl for each) into fluorescence-activated cell sorting (FACS) tubes and used for MP detection. The final MP suspension was used for the flow cytometric MP detection experiment.

The identification of MPs was determined by using a FACSria flow cytometer with Cell Diva software (Becton Dickinson). Briefly, 90 μl of MP suspension was incubated in the dark with 10 μl of the relevant monoclonal antibody (MAb) for 15 min at room temperature. Each of the MAbs was titrated, to ensure that there was no nonspecific binding due to excess staining and to ensure that there was no wastage of antibody (fluorescein isothiocyanate [FITC]-labeled annexin V for the presence of MPs, phycoerythrin [PE]-labeled anti-glycophorin A for erythrocyte-derived MPs, PE-labeled anti-CD31 for endothelial cell MPs, and PE-labeled anti-CD41a for platelet MPs obtained from Invitrogen and BD Biosciences), and then transferred to the special FACS-compatible Trucount tubes with a precounted number of beads, followed by thorough mixing. The Trucount beads were a part of the sample but exclusive from the MPs and did not have by-product, which was confirmed by adding 0.5 ml of the same buffer that was used to suspend the MP preparations for flow to the Trucount tubes. The data were collected using the same voltages that were used to collect the sample data. The results did not show any contaminants. There was no binding of any MPs to the Trucount beads. Sample data were acquired on the FACSria and analyzed for 50,000 events. MP gating was accomplished by preliminary standardization experiments (Fig. 1A), and events in the MP gate were further assessed for labeling with the specific fluorescence-labeled MAbs to distinguish the different specific cell-derived MPs (Fig. 1B, C, and D). The number of absolute MP counts of per ml was calculated as follows: absolute MP count = [(the number of positive events × the number of beads added)/(the number of beads counted × the plasma volume in μl)] × the dilution factor. The dot plots showed the Trucount beads on the region P1 gate and the MAb-labeled MPs on the region P2 gate (Fig. 1B, C, and D), a finding indicative of the presence of MPs from different cellular sources.

DNA isolation and genotyping. The human genomic DNA from all patients and HC were purified from whole blood using QIAamp blood DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The genotyping of −477C/T and −320G/C was performed by using the PCR/two-primer-pair method with primers described elsewhere (21). The single nucleotide polymorphism (SNP) at the −477 position in the promoter with a change in C/T was analyzed by an amplification of the 351-bp size fragment by PCR using the primers AB1 Forward (5'-CTCGGGTCCTCTGAGGGACCT-3') and AB1 Reverse (5'-CCGCAGACTCTCTATCCAC-3'). The final amplicon, consisting of a restriction site for the AclI enzyme (C/CGC) in the presence of the C allele, was identified by 148-, 130-, and 79-bp products detected using gel electrophoresis. The SNP at position −320 on the promoter with a G/C change at this site was analyzed by amplifying the 133-bp fragment described earlier (21) by using a pair of mismatch primers—AB2 Forward (5'-TGCTTGGCGTTCCTGAGGGAGATTC-3') and AB2 Reverse (5'-GGGCACCAGTGGAATTTGCTTCCTCTAGATC-3')—to generate a

FIG 1 Enumeration of MPs by flow cytometric analysis. (A) Complete MP counts. The P1 gate indicates the bead gate that includes Trucount beads used for enumerating MPs. The P2 gate indicates the MP gate determined by the forward-scatter and side-scatter (SSC) characteristics of light. (B) Erythrocyte-derived MPs detected by glycophorin-phycoerythrin (PE) labeling on the x axis in relation to SSC on the y axis. The P1 indicates the bead gate, and the P2 indicates the erythrocyte MP gate. (C) Detection of endothelial cell-derived MPs detected by CD31-PE labeling on the x axis in relation to SSC on the y axis. The P1 indicates the bead gate, and the P2 indicates the endothelial cell MP gate. (D) Detection of platelet-derived MPs detected by CD41a-PE labeling on the x axis in relation to SSC on the y axis. The P1 indicates the bead gate, and the P2 indicates the platelet MP gate.

restriction site for the BstYI enzyme (R/GATC) in the presence of the G allele. This was identified by 133-, 102-, and 31-bp products checked by illumination of the electrophoresed agarose gel. About 20% of the randomly selected samples were regentyped for -477C/T and -320G/C polymorphisms, and the results were found to be 100% concordant, which ensured the absence of any genotyping error.

Statistical analysis. Genotype and allele frequency were calculated by direct counting. The Fisher test was used to compare genotypes and allele frequencies and to determine the associations of combined genotypes with clinical and laboratory data. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated by using GraphPad Prism 5.01. For the results shown in Table 2, the allele and genotypes with higher frequency were selected as a reference ($\text{OR} = 1$), and the other ORs were calculated relative to that reference (Fisher exact test, 2×2 contingency tables). The result was considered to be statistically significant when the P value was found to be <0.01 (Bonferroni correction for two SNPs, $0.05/2 = 0.01$). Deviations from the Hardy-Weinberg equilibrium (HWE) were tested by using an online resource tool (<http://www.oege.org/software/hwe-mr-calc.shtml>). SNPalyze software (Dynacom) was used to perform haplotype analysis of the promoter SNPs studied. Distributions of plasma MP levels in genotypes were assessed by using the D'Agostino-Pearson omnibus normality test. Based on the results of the normality test, the association of genotype with plasma MP was analyzed by analysis of variance or the Kruskal-Wallis test, followed by an appropriate post test. GraphPad Prism 5.01 software was used for these statistical analyses.

RESULTS

Case description of the study subjects. In the present study, blood samples drawn from 428 malaria patients (UM, $n = 142$; CM, $n = 175$, MOD, $n = 71$; NCSM, $n = 40$) were genotyped for ABCA1 promoter polymorphisms at positions -477 and -320 . Of 428 samples, 128 fresh samples (UM, $n = 43$; CM, $n = 52$; MOD, $n = 21$; NCSM, $n = 12$) and 30 HC samples were selected to study the plasma level of MPs, and the patient characteristics of the 128 samples were not significantly different from those of the 428 samples. Table 1 provides anthropometric, clinical, and hematological characteristics for all of the subjects in the five study groups (CM, MOD, NCSM, UM, and HC). All malaria patients and control subjects were in the adult age group and were natives of Odisha, had permanent resident status, and had the same ethnic background. The clinical severity was uniformly spread over both genders and all age groups. There was no correlation between age and gender with blood parasitemia level in the study samples.

-477C/T and -320G/C promoter polymorphisms in malaria patients. The genotype and allele frequencies of the -477C/T and -320G/C polymorphisms in the promoter of the ABCA1 gene were studied in a cohort of 428 cases of malaria belonging to the CM, MOD, NCSM, and UM groups (Table 2), and genotyping was conducted successfully for all of the samples.

TABLE 1 Clinical characteristics of subjects in different study groups analyzed for ABCA1 promoter polymorphisms at positions –477 and –320^a

	Severe <i>P. falciparum</i> malaria clinical categories (SM)					
Parameter	CM	MOD	NCSM	UM	HC control	<i>P</i> ^b
Patient characteristics						
Total no.	175	71	40	142	30	NA
No. male/no. female	91/84	45/26	17/23	57/85	13/17	NA
Mean age (yr) for male/ female patients ^c	30.92 (15–47)/ 31.25 (15–47)	32.67 (16–47)/ 30.12 (15–41)	34.06 (21–45)/ 29.61 (16–43)	29.77 (15–42)/ 30.16 (15–47)	29.77 (15–42)/ 33.71 (16–33)	NS
Mean level ± SEM						
Hemoglobin (g/dl)	6.303 ± 0.1007	6.656 ± 0.166	6.750 ± 0.13	9.71 ± 0.15	11.5 ± 0.93	<0.0001
Parasitemia (count/μl)	88,092 ± 8,668	111,690 ± 14,954	78,594 ± 17,303	2,845 ± 17.65	0	<0.0001
No. of patients with:						
Schizontemia	37	21	19	0	0	<0.0001
Unarousable coma	175	0	0	0	0	<0.0001
Splenomegaly	99	71	31	45	0	<0.0001
Hepatomegaly	94	71	26	0	0	<0.0001
Pallor	175	71	40	66	0	<0.0001
Respiratory distress	0	17	12	0	0	<0.0001
Hemoglobinuria	0	26	7	0	0	<0.0001
Jaundice	0	47	21	0	0	<0.0001
Gastrointestinal hemorrhage	0	31	0	0	0	<0.0001
Acute renal failure	0	38	0	0	0	<0.0001
No. of samples:						
Tested for genotype C–477T	175	71	40	142	30	NA
Tested for genotype G–320C	175	71	40	142	30	NA
Tested for MP level	52	21	12	43	30	NA

^a CM, cerebral malaria; MOD, multiorgan dysfunction; NCSM, noncerebral severe malaria; UM, uncomplicated malaria; HC, healthy controls.^b Group comparisons were analyzed by the Kruskal-Wallis tests. NA, not applicable; NS, not significant. All values of <0.0001 are significant.^c Ranges are indicated in parentheses.

The distribution of the –477C/T and –320G/C polymorphisms in our study population did not display deviation from the HWE in each of the clinical groups tested. The prevalence of wild-type CC genotype of the –477 position was significantly lower in UM patients compared to CM (OR = 16.04), MOD (OR = 16.93), and NCSM (OR = 19.86) patients. Conversely, the prevalence of the mutant TT genotype of the –477 position was found to be significantly greater in UM patients than in CM (OR = 12.5), MOD (OR = 12.99), and NCSM (OR = 39.23) patients. In the –320G/C position, the wild-type GG genotype was significantly less prevalent in UM patients compared to CM (OR = 10.95), MOD (OR =

15.65), and NCSM (OR = 7.55) patients and, correspondingly, the mutant CC genotype was significantly more prevalent in UM patients compared to CM (OR = 19.67), MOD (OR = 24.26), and NCSM (OR = 6.82) patients (Table 2). There were no statistical differences in allele frequencies between cases and controls in each gender group for the SNPs tested (data not shown). The mutation frequencies for the –477 C/T and –320G/C polymorphisms in the UM and HC groups were comparable (data not shown).

Plasma levels of MP. Of the 428 cases investigated here, plasma samples from only 128 cases were available for quantification of the MPs. The total MP level was significantly higher in CM, MOD,

TABLE 2 Genotypic and allelic frequencies of the ABCA1 polymorphisms in the different malaria groups

Genotype	No. of subjects (%) ^a				OR (95% CI) and <i>P</i> ^b					
					CM vs UM		MOD vs UM		NCSM vs UM	
	CM	MOD	NCSM	UM	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
C–477T										
CC	45 (25.7)	19 (26.8)	12 (30)	3 (2.1)	16.04 (4.86–52.8)	<0.0001*	16.93 (4.8–59.6)	<0.0001*	19.86 (5.25–75.01)	<0.0001*
CT	102 (58.3)	41 (57.7)	18 (45)	39 (25.5)	3.69 (2.29–5.9)	<0.0001*	0.27 (0.15–0.50)	<0.0001*	2.16 (1.04–4.45)	0.05†
TT	28 (16)	11 (15.5)	10 (25)	100 (70.4)	12.5 (7.27–21.4)	<0.0001*	12.99 (6.21–27.14)	<0.0001*	39.29 (18.87–81.78)	<0.0001*
Allele	350	142	80	284						
C	192 (54.9)	79 (55.6)	42 (52.5)	45 (15.8)						
T	158 (45.1)	63 (44.4)	38 (47.5)	239 (24.2)	6.45 (4.40–9.45)	<0.0001*	6.66 (4.2–10.54)	<0.0001*	5.87 (3.14–10.1)	<0.0001*
G–320C										
GG	57 (32.6)	29 (40.9)	10 (25)	6 (4.2)	10.95 (4.55–26.3)	<0.0001*	15.65 (6.08–40.26)	<0.0001*	7.55 (2.54–22.4)	0.0003*
GC	106 (60.6)	38 (53.5)	23 (57.5)	52 (36.6)	2.65 (1.68–4.19)	<0.0001*	1.99 (1.11–3.55)	0.026†	2.34 (1.14–4.78)	0.028†
CC	12 (7)	4 (5.6)	7 (17.5)	84 (59.2)	19.67 (10.01–38.64)	<0.0001*	24.26 (8.37–70.24)	<0.0001*	6.82 (2.82–16.49)	<0.0001*
Allele	350	142	80	284						
G	220 (62.9)	96 (67.6)	43 (53.8)	64 (22.5)						
C	130 (37.1)	46 (32.4)	37 (46.2)	220 (77.5)	5.81 (4.08–8.27)	<0.0001*	7.17 (4.58–11.23)	<0.0001*	3.99 (2.37–6.72)	<0.0001*

^a CM, cerebral malaria; MOD, multiorgan dysfunction; NCSM, noncerebral severe malaria; UM, uncomplicated malaria. Allele counts are equal to twice the total numbers for C–477T and G–320C, respectively.^b *P* values were determined by the Fisher exact test. *, Significant; †, not significant.

FIG 2 Total and cell-specific MP levels per μ l of plasma on admission in the different clinical groups of malaria. CM, cerebral malaria; MOD, multiorgan dysfunction; NCSM, noncerebral severe malaria; UM, uncomplicated malaria. Comparisons between clinical groups were calculated by Mann-Whitney test. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

and NCSM patients compared to UM and HC subjects, as shown in Fig. 2, and the MP levels in the UM and HC groups were comparable. The levels of erythrocyte MPs, platelet MPs, and endothelial MPs showed a similar trend in significance in each group of SM patients compared to the UM and HC groups, and we observed that the erythrocyte MPs were most prevalent in all groups compared to endothelial cell MPs and platelet MPs (Fig. 2), followed by the number of endothelial cell MPs and then platelet MPs per μ l of plasma from the malaria patients.

Effect of genotypes on the plasma MP level. The association of the $-477C/T$ polymorphism to the plasma MP level was assessed (Fig. 3A). There was a trend toward higher plasma level of MP in subjects with a homozygous wild (CC) genotype in the CM, MOD, and NCSM groups compared to subjects with a heterozygote (CT) genotype or a homozygous mutant (TT) genotype (Fig. 3A). The MP levels in the different genotypic groups (CC, CT, and TT) were compared, and the differences observed were significant in CM patients ($P = 0.0051$) and MOD patients ($P = 0.0234$), whereas the levels of MPs were not significantly different in NCSM and UM patients.

The association between plasma MP and the $-320G/C$ polymorphism revealed that the GG wild genotypes had higher levels of MPs in CM, MOD, NCSM, and UM patients compared to heterozygous GC and mutant CC genotype individuals (Fig. 3B). Analyzing the levels of MPs in the different genotypic groups (GG, GC, and CC) revealed the levels in CM patients to be highly significant ($P < 0.0001$) and also significant ($P = 0.112$) in MOD patients, whereas in NCSM and UM patients they were not significantly different.

Haplotypes. SNPalyze software was used to perform the haplotype analysis, and it helped in the construction of three major haplotypes deriving from the two promoter SNPs studied. The haplotypes T-C and C-G were found to be in significant difference in CM, MOD, and NCSM patients compared to UM patients (Table 3). The haplotype T-G was not determined to be significantly different by the program in CM, MOD, and NCSM patients compared to UM patients (Table 3). Pairwise linkage disequilibrium (LD) analysis of the two loci showed that these are in LD, with $|D'| = 1$ for the CM and UM pair, $|D'| = 1$ for the MBL NCSM and UM pair, and $|D'| = 0.9655$ for the MOD and UM pair. These findings suggest that the two promoter polymorphisms are highly associated with malaria severity and a higher MP level.

DISCUSSION

This is the first report of an association between ABCA1 gene promoter polymorphisms with the plasma MP level in human malaria patients, thereby giving some idea regarding the functional relevance of the former in clinical manifestations of human malarial infections. Our findings are consistent with the notion that ABCA1 gene variations may contribute to interindividual variability in susceptibility to malaria and disease severity and indicate an association of $-477C/T$ and $-320G/C$ polymorphisms with an increase in plasma MP level and disease manifestation in cases of malaria in humans.

The efflux of MPs from cells is variably regulated by ABCA1 gene, where the deletion of the same has been seen to confer protection against rodent CM (6), and this gene is also responsible for

FIG 3 Association between ABCA1 promoter polymorphisms and plasma MP level in different clinical groups of malaria. Abbreviations are as defined for Fig. 2. The distribution of data of plasma MPs in the different categories of malaria patients segregated according to the genotype was assessed by using the D’Agostino-Pearson omnibus normality test and analyzed with the Kruskal-Wallis test, followed by a Dunn’s multiple-comparison test, with a *P* of <0.05 considered significant. (A) Association of plasma MP with –477C/T variant in the ABCA1 promoter. CC, homozygous wild; CT, heterozygous; TT, homozygous mutant. (B) Association of plasma MP and –320G/C variant in the ABCA1 promoter. GG, homozygous wild; GC, heterozygous; CC, homozygous mutant.

overturning the phosphatidylserine (PS) from the inside to the outside leaflet of the cells (28). The regulation of this feat ultimately leads to different levels of receptiveness to MP levels. Since ABCA1 is involved in PS exposure in vesiculation, changes in ABCA1 expression should have impact on perturbations in the

MP profiles and affect disease predisposition. Therefore, the significance of the ABCA1 gene in disease pathogenesis was investigated here by focusing on its promoter region since this region encompasses the regulatory factors for gene expression. The –477C/T and –320G/C polymorphisms in the promoter of the

TABLE 3 Haplotype frequencies in different malaria groups

Haplotype	No. of subjects (%) ^a				OR (95% CI) and <i>P</i> ^b					
					CM vs UM		MOD vs UM		NCSM vs UM	
	CM	MOD	NCSM	UM	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
T-C	65 (37)	23 (32)	17 (43)	109 (77)	5.5 (3.4–9.1)	<0.0001*	2.4 (1.1–5.1)	<0.0001*	4.4 (2.1–9.3)	<0.0001*
C-G	96 (54)	39 (55)	20 (49)	21 (15)	7.0 (4.0–12.1)	<0.0001*	7.02 (3.6–13.5)	<0.0001*	5.7 (2.6–12.4)	<0.0001*
T-G	12 (7)	8 (11)	1 (3)	9 (6)	1.08 (0.44–2.66)	NS	1.8 (0.69–5.09)	NS	0.37 (0.04–3.08)	NS

^a CM, cerebral malaria; MOD, multiorgan dysfunction; NCSM, noncerebral severe malaria; UM, uncomplicated malaria.

^b *P* values were determined by the Fisher exact test. *, Significant; NS, not significant.

ABCA1 gene, earlier identified in coronary atherosclerosis (21), showed a defect in reverse cholesterol transport, thereby causing increased cholesterol accumulation in the vessel wall and an increased atherosclerotic burden. We sought to determine whether these polymorphisms in the ABCA1 gene cause any alteration in the amount of MP efflux from the cells in human malaria patients compared to HC and to assess the functional relevance of these polymorphisms, if any, in human malaria.

The patients in the present study who carried the C allele of the –477C/T polymorphism were more likely to suffer from SM. The results of Lutucuta et al. (21) and Pasdar et al. (30), who had studied this polymorphic position in the promoter of the gene, showed that arteriosclerosis patients with the mutant allele T were more likely to have severe arteriosclerosis. Another study conducted among a Japanese population (31) on the same polymorphism did not find any association of the SNP with the coronary artery disease. The mutated T allele in our study was found in significantly higher numbers in UM patients than in CM, MOD, and NCSM patients. The MP plasma levels in the patients studied here were highest in CM, MOD, and NCSM patients compared to UM patients.

When plasma MP levels were analyzed according to the genotypes of the patients, it was observed that –477C/T was associated with plasma MP levels in malaria patients that significantly increased in the order TT < CT < CC in our study. The levels of specific cell MPs in circulation exhibited a similar trend in the level of erythrocyte MPs, platelet MPs, and endothelial MPs in patients segregated according to the various genotypes. Therefore, we inferred that the C allele of the –477C/T polymorphism was responsible for higher levels of plasma MP in CM, MOD, and NCSM patients compared to UM patients, thereby showing the T allele to have a protective effect against human SM.

The –320G/C polymorphism in the promoter of the ABCA1 gene also showed an association with the manifestation of the disease characteristics in the patients. An earlier arteriosclerotic study (20) focusing on this SNP did not find any association with the severity of the disease in the patients. The wild-type G allele of this SNP was found to be highly associated with the severity of the disease in CM, MOD, and NCSM patients against very low numbers in UM patients. The functional relevance of this polymorphism was analyzed by associating the same polymorphism with the plasma MP levels in the patients. The MP levels in patients with homozygous GG genotypes were highest for the CM, MOD, and NCSM patients and lowest for UM patients with the GG genotype. The cell-specific MPs also showed higher levels in CM, MOD, and NCSM patients with GG genotypes and lower in UM patients with a similar genotype. The mutated CC genotype is influential in being protective in populations for which malaria is

endemic. Haplotype analysis showed the haplotype T-C to be more common in UM patients ($P < 0.0001$) than in CM, MOD, and NCSM patients, whereas haplotype C-G was more common in CM, MOD, and NCSM patients ($P = 0.0001$) compared to UM patients. Haplotype T-G was detected in a very small proportions of CM, MOD, NCSM, and UM patients ($P =$ not significant).

The T allele of the –477C/T polymorphism and the C allele of the –320G/C polymorphism were associated with decreased MP levels, which is consistent with the finding that the two polymorphisms are significantly more prevalent in UM patients than in CM, MOD, and NCSM patients. The mouse model study (6) had indicated that a lower circulating MP level confers resistance to CM; therefore, this gene may have evolved and played a significant role in protection against SM in the Orissa population by decreasing the efflux of MPs into the circulation. The malaria patients represented a cross-section of the population for the geographical area, and the patients developed the diseases and aberrant MP levels if they harbored genotypes that predisposed them to such a development. In contrast, UM patients with low MP levels should have “good” mutated variations that accumulate and protective effects to against the development SM, as well as normal or low MP levels. There is no previous recorded study linking ABCA1 promoter polymorphism and plasma MP level in human malaria and the antagonistic result in American coronary arteriosclerosis patients (30) and no association in the Japanese population (31); thus, it appears that the genotypic effects of ABCA1 may be also be influenced by other factors such as genetic backgrounds and environmental factors.

In conclusion, our findings suggest that the ABCA1 gene promoter –477T and –320G polymorphisms and the T-G haplotype have a protective effect against SM. The results also indicate that MP production is boosted in febrile patients during natural *P. falciparum* infection and that the –477C and –320G polymorphisms in the ABCA1 gene promoter lead to increased MP production. Taken together, our findings here indicate a protective effect of the mutant genotype of the ABCA1 gene promoter in determining low MP production during malarial infection, which eventually influences the severity of malaria infection in humans.

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