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Gene Expression Analysis Reveals Genes Common to Cerebral Malaria and Neurodegenerative Disorders

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Cerebral malaria, a reversible encephalopathy affecting young children, is a medical emergency requiring urgent clinical assessment and treatment. We performed a whole-transcriptomic analysis of blood samples from Malian children with cerebral or uncomplicated malaria. We focused on transcripts from pathways for which dysfunction has been associated with neurodegenerative disorders. We found that *SNCA*, *SIAH2*, *UBB*, *HSPA1A*, *TUBB2A*, and *PINK1* were upregulated (fold-increases, ≥2.6), whereas *UBD* and *PSMC5* were downregulated (fold-decreases, ≤4.39) in children with cerebral malaria, compared with those with uncomplicated malaria. These findings provide the first evidence for pathogenic mechanisms common to human cerebral malaria and neurodegenerative disorders.

Keywords. cerebral malaria; human; PBMCs; mRNA; expression; neurodegenerative disorders; common pathways.

Cerebral malaria (CM), one of the most frequent severe complications of *Plasmodium falciparum* infection, is a diffuse reversible encephalopathy characterized by seizures and a loss of consciousness. It mostly occurs in young children and is a major cause of death. One third of survivors develop epilepsy or other neurological sequels. CM is thought to result from the sequestration of parasites in the small blood vessels of the brain and the dysregulation of key immune system elements, with high levels of inflammation, breakdown of the blood-brain barrier, and brain swelling. Host genetic determinants make a major contribution to disease severity and the outcome of infection. However, the cellular and molecular regulatory mechanisms underlying the pathogenesis of CM are not fully understood. Knowledge of these mechanisms would lead to improvements in diagnosis and treatment and better clinical outcomes. Analyses of differential gene expression constitute a powerful approach for identifying suitable biological indicators for predicting the eventual outcome of *P. falciparum* infection, making it possible to provide appropriate treatment more rapidly. Recent complementary DNA microarray studies have shown significant differences between the gene expression profiles of CM-resistant and CM-susceptible mice [1]. In humans, transcriptomic analyses of RNA extracted from the peripheral blood mononuclear cells (PBMCs) of malarial patients infected with *P. falciparum* revealed that changes in the expression of human innate immune pathway genes were correlated with the severity of malaria [2]. Whole-blood transcriptomic analyses have also provided insight into pathogenesis and have been used to identify biomarkers of neurodegenerative and psychiatric disorders [3] or of other infectious diseases, such as leprosy.

The aim of this study was to use blood-based gene expression profiles to identify biological pathways and molecules potentially reflecting the pathological mechanisms of CM. We used a gene selection strategy combining expression profiling and pathway analysis to focus on genes displaying changes in expression also known to be associated with neurological disorders. We show here, for the first time, that CM and neurodegenerative disorders have pathogenic mechanisms in common.

METHODS

This study was approved by the local ethics committees of the Faculty of Medicine, Pharmacy, and Odonto-Stomatology of the University of Bamako and by French ethics committees, including those of INSERM, the Comité de Protection des Personnes (protocol 212 CO2), the Ministère de l'Enseignement Supérieur et de la Recherche (protocol DC-2011-1426), and the Commission Nationale de l'Informatique et des Libertés (protocol 1564177). All experimental methods were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all parents.

Patients

CM was defined according to World Health Organization criteria as a state of unarousable coma with a Blantyre coma scale score of <2, a hematocrit of >16%, and parasitemia with asexual stages of *P. falciparum*. Meningitis was ruled out by lumbar puncture. Subjects with uncomplicated malaria (UM) had a thick blood film positive for *P. falciparum*, a Blantyre coma scale score of 5, and a hematocrit of >26%. The children with UM had never developed CM. Malian children were recruited through the Pediatrics Department of Gabriel Toure Hospital in Bamako, as part of a larger prospective field study.

In this study, we analyzed gene expression in 13 patients with CM (male to female ratio, 7:6; mean age [±SD], 6.2 ± 3.8 years)

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and 12 patients with UM matched for age and sex (male to female ratio, 6:6; mean age [±SD], 6.5 ± 3.6 years). Blood samples were collected before the treatment of the children.

RNA Extraction

A peripheral blood sample was collected from each participant, and peripheral blood mononuclear cells (PBMCs) were isolated within 1 hour after sample collection. Total RNA was extracted with TRIzol reagent (Life Technologies) according to the manufacturer's instructions but with an additional purification step performed with the RNA Clean and Concentrator kit (Zymo Research). The quality of total RNA was assessed with an RNA LabChip (Agilent 2100 Bioanalyzer, Agilent). Only samples with an RNA integrity number of >8 were used for further analysis.

Whole-Transcriptome Analysis

The samples of high-quality RNA were marked with Cy3 fluorescent dye, and then they were hybridized using the SurePrint G3 Human Gene Expression 8 × 60K microarray kit, version 1 (Agilent), according to the manufacturer's instructions. The slides were composed of 8 high-definition 60-mer oligomicroarrays. Each microarray contained about 22 700 genes based on RefSeq and 7419 large intergenic noncoding RNAs. Fold-changes and *P* values were calculated for each gene, using Genespring Software (Agilent). The moderated *t* test and the Benjamini-Hochberg correction were used to obtain the *P* value. Pathway analysis was also performed with Genespring Software.

Validation of Gene Expression Data by Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis

We reverse-transcribed 400 ng of total RNA to generate complementary DNA. The expression of selected genes was analyzed by TaqMan PCR, on an ABI 79000 real-time PCR thermocycler (Life Technologies), according to the manufacturer's instructions. The data were normalized with 2 housekeeping genes (*RPLP0* and *GAPDH*). Data were analyzed by the ΔΔCt method ($2^{-\Delta\Delta Ct}$), with Thermo-Fisher software (cloud).

RESULTS

We characterized the biological pathways dysregulated during CM by analyzing the transcriptomes of 7 children with CM and 8 children with UM who were selected at random. In total, 2900 probe sequences differed significantly between the CM and UM groups, with an absolute fold-change of >1.1 and a *P* value of <.05, even after Benjamini-Hochberg correction (Table 1). In total, 436 probe sequences were identified as differentially expressed, yielding an absolute fold-change of >3 (Table 1): 403 of these sequences were upregulated and 33 downregulated in children with CM. GeneSpring pathway analysis revealed that a number of different Wiki biological pathways were significantly associated with the disease (Table 2). Remarkably, several genes from pathways associated with neurodegenerative disorders were substantially overexpressed or underexpressed in patients with CM relative to those with UM (Table 2). In particular, 18 genes from the parkin-ubiquitin proteasomal system, 14 from the proteasome degradation pathway, and 6 from the Parkinson disease pathway were found to be differentially expressed between the 2 groups. Six genes from these 3 pathways were significantly more strongly expressed in children with CM than in those with UM, with fold-increases of >2: *SNCA, SIAH2, UBB, HSPA1A, TUBB2A*, and *PINK1* (Table 3). Four genes, *UBD, UBE1L, PSMC5*, and *PSMD5*, were downregulated in children with CM, with fold-decreases of ≤4.39 (Table 3).

We investigated whether the changes in expression observed on microarray analysis for these 10 selected genes could be confirmed in a larger sample set of 13 children with CM and 12 with UM. Real-time PCR validation was performed in duplicate and confirmed the significant difference in expression levels for 8 genes. The relative expression levels ($2^{-\Delta CT}$) for these genes are

Table 1. Total Number of Genes Differentially Expressed Between Children With Cerebral Malaria and Those With Uncomplicated Malaria, According to Absolute Fold-Changes in Expression and *P* Values for Differences in Expression

Absolute Fold-Change	*P* < .05	*P* < .02	*P* <. 01	*P* < .005	*P* < .001
>1.1	2900	1356	724	356	63
>1.5	2807	1353	724	356	63
>2.0	1366	848	538	310	63
>3.0	436	339	246	167	39

P values were determined after Benjamini-Hochberg correction for multiple testing, with values of <.05 considered statistically significant.

Table 2. Partial Results of Pathways Analysis

Pathway	No. of Differentially Expressed Genes	Total No. of Genes	*P*
Parkin-Ubiquitin Proteasomal system_WP2359_53587	18	73	2.9×10^{-6}
Proteasome Degradation_WP183_45274	14	65	2.7×10^{-4}
Mitochondrial Gene Expression_WP391_48245	7	19	3.3×10^{-4}
Senescence and Autophagy_WP615_47912	19	106	3.9×10^{-4}
Glycolysis and Gluconeogenesis_WP534_51732	10	49	.0034
Synthesis and Degradation of Ketone Bodies_WP311_43510	3	5	.0039
Inflammatory Response_WP453_41201	7	33	.0064
Spinal Cord Injury_WP2431_55909	15	102	.008
Tryptophan Metabolism_WP465_43616	8	47	.022
Parkinson_Disease_WP2371_29836	6	38	.043

The WikiPathways in which the genes were known to be involved are listed. We show only the pathways containing at least 3 dysregulated genes and known to be involved in neurodegenerative disorders or identified in gene expression analysis in a mouse model of malaria.

Table 3. Genes Related to the Parkin, Parkinson, and Proteasome Degradation Pathways Displaying Differential Expression Between Cerebral Malaria and Uncomplicated Malaria in Children

Pathway(s)	Gene	Probe Name	Absolute Fold-Change	Regulation Status	*P*[a]	FDR[b]
Parkin_WP2359_53587	*HSPA1A*	A_23_P111132	3.27	Upregulated	.028	.044
Parkin_WP2359_53587, Parkinson_WP2371_29836	*SNCA*	A_23_P29939	2.92	Upregulated	.028	.044
Parkin_WP2359_53587	*SIAH2*	A_33_P3358342	2.85	Upregulated	.0044	.039
Parkinson_WP2371_29836	*PINK1*	A_23_P23194	2.59	Upregulated	.013	.041
Parkin_WP2359_53587	*TUBB2A*	A_33_P3365002	2.31	Upregulated	.028	.044
Parkinson_WP2371_29836, Proteasome_WP183_45274	*UBB*	A_23_P27215	2.11	Upregulated	.04	.047
Proteasome_WP183_45274	*UBD*	A_23_P81898	4.39	Downregulated	.010	.040
Proteasome_WP183_45274, Parkinson_WP2371_29836	*UBE1L* (*UBA7*)	A_23_P21207	1.87	Downregulated	.0068	.039
Proteasome_WP183_45274, Parkin_WP2359_53587	*PSMC5*	A_23_P164035	1.59	Downregulated	.012	.041
Proteasome_WP183_45274, Parkin_WP2359_53587	*PSMD5*	A_33_P3305254	1.59	Downregulated	.015	.042

[a]By the moderated *t* test, with values of <.05 considered statistically significant.

[b]The false-discovery rates (FDRs) represent significant results (defined as those with *P* values of <.05) after Benjamini-Hochberg correction for multiple testing.

Abbreviations: *HSPA1A*, encodes heat shock 70-kD protein 1; *PINK1*, encodes PTEN-induced putative kinase 1; *PSMC5*, encodes proteasome 26S subunit ATPase 5; *PSMD5*, encodes proteasome 26S subunit non-ATPase 5; *SIAH2*, encodes SIAH E3 ubiquitin protein ligase family member 1; *SNCA*, encodes α-synuclein; *TUBB2A*, encodes tubulin β2A; *UBB*, encodes ubiquitin B; *UBD*, encodes ubiquitin D; *UBE1L* (*UBA7*), encodes ubiquitin activating enzyme 7.

presented in Figure 1. The difference in expression between subjects with CM and those with UM, expressed as a fold-increase, was 3.2 for *SNCA* (*P* = .026), 3.1 for *SIAH2* (*P* = .001), 2.6 for *UBB* (*P* = .004), 2.7 for *HSPA1A* (*P* = .013), 3.2 for *TUBB2A* (*P* = .009), and 3.2 for *PINK1* (*P* = .001), whereas for *UBD* and *PSMC5*, we obtained a fold-decrease of 6 (*P* = .001) and 1.5 (*P* = .01), respectively. We then analyzed the relationship between the expression levels of these genes in patients with CM and those with UM. We found significant positive correlations between expression levels for each pair-wise comparison between *UBB, SIAH2, SNCA, TUBB2A*, and *PINK1* (*P* < .01; Pearson correlation, >0.8). We found significant negative correlations between expression levels in children for *UBD* and *PINK1* (*P* = .01; Pearson correlation, – 0.646) and for *UBD* and *SIAH2, SNCA, TUBB2A*, and *UBB* (*P* < .05; Pearson correlation, less than –0.4).

Discussion

In this study, we identified human genes and pathways involved in CM, suggesting possible targets for drug development. The key findings of this study are (1) the efficacy of this approach for identifying changes in the levels in human peripheral blood of transcripts involved in both CM and neurological disorders, Parkinson disease, and Alzheimer disease; (2) the overlap between the pathways dysregulated in both the human and mouse model of CM, such as glycolysis/gluconeogenesis, tryptophan metabolism, immune responses, and brain function [1]; and (3) the confirmation that the molecular profiling of PBMCs can reflect physiological and pathological events in the brain, as previously suggested by Mohr et al [4].

We focused here on genes and pathways that have been clearly implicated in neurological disorders: the interconnected parkin, Parkinson, and proteasome pathways, in particular. These pathways were considered to be of particular interest because they had never before been studied in human malaria, unlike immune response pathways [2, 5]. We showed that *SNCA, SIAH2, HSPA1A, TUBB2A, PINK1*, and *UBB* were expressed significantly more strongly in patients with CM than in patients with UM, whereas *UBD* and *PSMC5* from the proteasome degradation pathway were expressed less strongly. Our findings suggest that transcriptomic analyses of peripheral blood can provide important insights into pathogenic mechanisms affecting the brain. These findings are entirely consistent with those of Liew et al [6], indicating significant similarities in gene expression between the blood and brain (>80%) and showing that the profiling of gene expression in the blood could be used for diagnostic and prognostic purposes.

SNCA, which encodes α-synuclein, a protein expressed predominantly in the brain, has been identified as the major causal gene in several neurodegenerative disorders. There is growing evidence to suggest that the overproduction and aggregation of this protein lead to brain dysfunction that is sufficient to cause disease [7]. We found that *SNCA* was overexpressed in children with CM, suggesting a possible deleterious role of this gene in this disease. Another gene from the same pathway, *SIAH2*, was also overexpressed in children with CM. This gene encodes an E3 ubiquitin-conjugating enzyme that plays a critical role in the hypoxia response [8], which is frequently observed in patients with CM. Under hypoxia, *SIAH2* messenger RNA

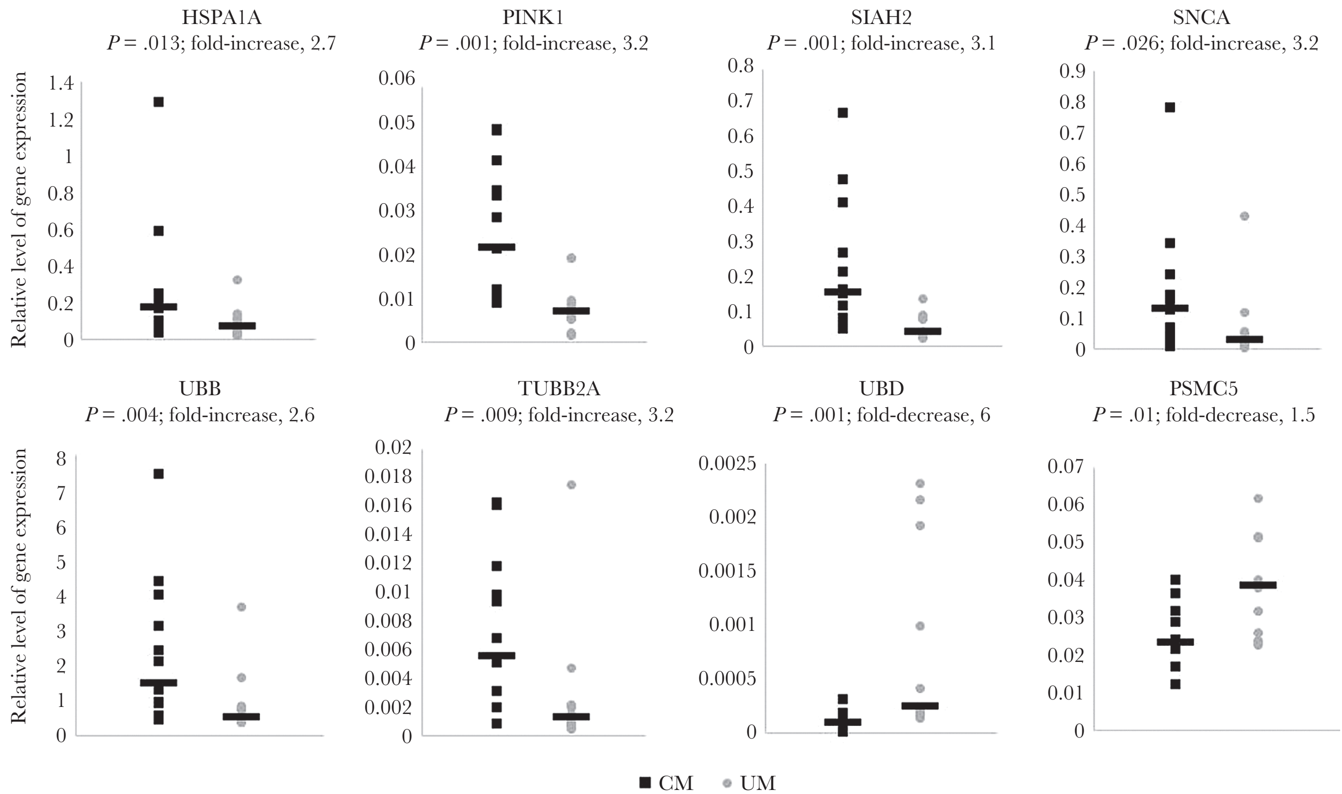

Figure 1. Real-time polymerase chain reaction–based validation of messenger RNA levels for the 8 most significantly dysregulated genes from the three selected pathways (parkin, Parkinson and proteasome degradation). Samples from 13 children with cerebral malaria (CM) and 12 with uncomplicated malaria (UM) were analyzed. Relative expression levels were calculated from $2^{-\Delta Ct}$ values. Values for children with CM are represented by black squares, and values for children with UM are represented by gray circles. The horizontal lines indicate median values. We used Student *t* tests to compare the results for the CM and UM groups.

levels increase and the encoded protein interacts with α-synuclein, promoting its aggregation, which is toxic. The formation of these aggregates is prevented by the suppression of endogenous *SIAH2* expression. PTEN-induced kinase 1 (PINK1) is a mitochondrial kinase that is strongly expressed in the brain and promotes cell survival, particularly under conditions of oxidative stress. It accumulates on the surface of defective mitochondria and recruits parkin, thereby promoting the selective degradation of dysfunctional mitochondria. Abnormal *PINK1* expression may be a causal factor in the development of mitochondrial dysfunction leading to neurodegenerative and neuroinflammatory disorders [9]. Despite its demonstrated protective effects, *PINK1* has been shown to be present at abnormally high levels in the brains of individuals with Alzheimer disease or multiple sclerosis [10], as observed in the blood of children with CM. *TUBB2A*, which encodes β-tubulin, was also overexpressed during CM. Mutations of this gene cause infantile-onset epilepsy and are associated with impaired brain development in humans, but the underlying pathogenic mechanism is unknown [11].

Molecular chaperones and the ubiquitin proteasome system are the first and second lines of defense against protein misfolding and aggregation. HSP-70, encoded by *HSPA1A*, is a chaperone protein that plays an important role in the refolding of misfolded proteins and the targeting of proteins for proteasomal degradation. A loss of Hsp70 activity has been associated with neurodegeneration and the formation of amyloid deposits of α-synuclein in Parkinson disease. However, the ability of HSP-70 to inhibit the aggregation of α-synuclein depends on factors such as nucleotide binding and the presence of the Hip cochaperone. In the presence of ATP (or ADP), Hsp70 overexpression may reduce α-synuclein toxicity but without preventing the highly cytotoxic accumulation of amyloid aggregates in tissue [12]. We found that *HSPA1A* was overexpressed in children with CM, as in patients with epilepsy, in whom HSP-70 levels are correlated with the duration and intensity of seizures, suggesting that this protein may be a marker of seizure-related brain injury [13]. Inflammation and oxidative stress, characteristic features of CM, may account for the increase in *HSPA1A* transcript levels in this disease and may reflect the severity of the injury. Nevertheless, further studies are required to determine whether HSP-70 is a cause or a consequence in the pathogenesis of CM. The ubiquitin proteasome system is an intracellular protein degradation system that plays an important role in maintaining intracellular homeostasis [14, 15]. The impairment of this system leads to

protein accumulation, resulting in neurodegenerative diseases, such as Alzheimer disease and Parkinson disease. The upregulation of *UBB* and the downregulation of *UBD* and *PSMC5* in children with CM suggest that the ubiquitin proteasome system is disturbed. Exogenous stress, mitochondrial alterations, and α -synuclein overexpression may also promote disruption of the ubiquitin proteasome system, with potentially protein aggregates.

In conclusion, our results suggest that CM has pathogenic mechanisms in common with other brain disorders involving protein aggregation. They also show that transcriptomic analysis of PBMCs is an efficient method for investigating changes in the brain. The identification of new molecules involved in CM pathogenesis could improve diagnostic capabilities or facilitate the development of new treatments.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. 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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