Autoantibody profile of patients infected with knowlesi malaria

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A B S T R A C T

Background: Autoantibodies or antibodies against self-antigens are produced either during physiological process-es to maintain homeostasis or pathological process such as trauma and infection. Infection with parasites includ-ing Plasmodium has been shown to generally induce elevated self-antibody (autoantibody) levels. Plasmodium knowlesi is increasingly recognized as one of the most important emerging human malaria in Southeast Asia that can cause severe infection leading to mortality. Autoimmune-like phenomena have been hypothesized to play a role in the protective immune responses in malaria infection.
Methods: We studied the autoantibody profile from serum of eleven patients diagnosed with P. knowlesi. Autoantigen arrays were used to elucidate the autoantibody repertoire of P. knowlesi infected patients. The patented OGT Discovery Array with 1636 correctly folded antigen was employed.
Results: Analysis of the patient versus control sera gave us 24 antigens with high reactivity with serum antibodies.
Conclusions: Understanding the autoantibody profile of malarious patients infected with P. knowlesi would help to further understand the host–parasite interaction, host immune response and disease pathogenesis. These reactive antigens may serve as potential biomarkers for cases of asymptomatic malaria and mild malaria or predictive markers for severe malaria.

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1. Introduction

Autoantibodies (AAbs) are antibodies to self-antigens that may ap-pear during physiological and pathological processes. It is believed that AAbs are generated through overexpression, mutation, release of proteins from damaged tissues, and misfolding or mispresentation of proteins, leading to their recognition by the immune system [1]. How-ever, under normal conditions, natural IgG AAbs do react with a variety of self-antigens in normal serum [2], with speculated roles in the estab-lishment of homeostasis, maintenance of self-tolerance and regulation of the immune response [3,4].

Autoantibodies (AAbs) have been implicated in parasitic infection such as trypanosomiasis, schistosomiasis, and malaria [5]. Numerous AAbs against self-antigens such as single-stranded DNA [5], smooth muscle [6], lymphocytes [7], phospholipids [8], brain beta tubulin III [9], erythrocytes [10], dendrites of neurons [11] and many others were found in patients with acute or chronic malaria infection. However, much remains to be known about the mechanisms involved in the dy-namics of autoimmunity and malaria.

Malaria remains a major health concern whereby almost half of the world's population is at risk. Out of the five human malaria parasite,

falciparum malaria is a major cause of malaria-associated morbidity and mortality [12]. It was not until a decade ago that Plasmodium knowlesi began gaining increasing recognition as the emergent human malaria in Southeast Asia [13]. Transition from mild malaria to severe malaria which often leads to fatal outcome can be sudden and requires immediate intervention. Intriguingly, cases of severe malaria caused by Plasmodium falciparum, Plasmodium vivax and P. knowlesi display similar patterns of inflammation and host responses [14–16].

To gain a better understanding of disease pathogenesis, host–pathogen interactions and host immune response, we utilized an autoantigen array to characterize the AAb repertoire of knowlesi-infected patients. The OGT Discovery Array utilizes correctly folded pro-teins that have the ability to display native, discontinuous epitopes [17] to identify specific AAb markers. Proteomic AAb profiling also provides a platform for recognizing and defining the signature serum AAbs of a dis-ease [18], some of which may be potential biomarkers. With that, we identified antigens with high reactivity with serum antibodies after analysis of the patient versus control sera.

2. Material and methods

2.1. Sample selection and collection

Blood from febrile patients suffering from symptoms of malaria were sent to the Parasites: South East Asian Diagnostic (Para:SEAD)

laboratory in separate EDTA and plain tube. Malaria infection was confirmed via microscopic examination on thin and thick blood smear. This is followed by a nested PCR using DNA extracted from blood of malaria infected patient for species specific identification. At the same time, serum was collected from the plain tube and stored at −20 °C until further use. For this study, 11 serum samples were collected from patients with PCR and microscopically confirmed *P. knowlesi* malaria infection. Blood from age and sex matched, healthy individuals (n = 11) with no malaria infection were taken and used as controls (Table 1). Ethical clearance was obtained from the University of Malaya Medical Centre Medical Ethics Committee.

2.2. OGT Discovery Array

The OGT protein array consists of 1636 immobilized, full-length, and correctly folded proteins. The proteins are immobilized on the array via an affinity tag, specifically the biotin carboxyl carrier protein (BCCP) domain of the *Escherichia coli* acetyl CoA carboxylase, reducing the possibility of affecting protein folding and function. Hence, each protein is expressed in insect cells as a fusion protein with a proprietary BCCP tag that monitors correct folding [17] and reduces nonspecific binding to a minimal level.

The following procedures were performed as a service provided by Sengenics Sdn Bhd, Malaysia. Serum sample (22.5 μl) was pipetted into 4.5 ml of serum assay buffer (SAB) containing 0.1% *v/v* Triton and 0.1% *w/v* BSA in PBS and vortexed to mix. The OGT array slide was placed in a slide box rack containing cold SAB and shaken at 50 rpm for 5 min. After washing, the array slide was placed in a slide hybridization chamber with an individual serum which had been diluted earlier. All slides were incubated on a horizontal shaker at 50 rpm for 2 h at 20 °C. The protein array slide was then rinsed twice in individual Pap jars with SAB, followed by another wash in the slide staining box for 20 min on the shaker at 50 rpm at room temperature.

2.3. Incubation with Cy3-anti IgG

Binding of IgG was detected by incubation of the array slide with Cy3-rabbit anti-human IgG (Dako Cytomation), labeled according to the manufacturer's recommended protocols. Arrays were immersed in hybridization solution containing a mixture of Cy3-rabbit anti-human IgG solution diluted to 1:1000 in SAB buffer for 2 h at 50 rpm in 20 °C. After incubation, the slide was dipped in 200 ml SAB buffer 3 times for 5 min at 50 rpm at room temperature. Excess buffer was removed by immersing the slide in 200 ml pure water. Slides were then dried for 2 min at 240 *g* at room temperature and stored at room temperature until scanning.

2.4. Visualization of protein spot and spot segmentation

Hybridization signals were measured with a microarray laser scanner (Agilent Scanner) at 10 μm resolution. Data sorting and analysis were done by customized computer scripts, whereas each spot was plotted using Agilent Feature Extraction software. Spot segmentation was performed as a semi-automatic quality control process in order to produce a viable result. In order to identify and align the spots properly, SpotFinder (TIGR), spot aligner software was used. The program takes the microarray images as inputs and produces a file consisting of relative frequency unit (RFU) values for all the spots and their background in the array. The output file is processed by subtracting the median RFU by taking into account the background median RFU. A median RFU value was chosen in order to negate the effects of outliers between the 4 protein replicates on the array.

2.5. Statistical analysis

Outliers were identified using principal component analysis (PCA) and removed. For each protein, the mean fold change of all cases was divided over mean fold change of all controls, resulting in an overall fold change. Then, the median fold change was compared with the fold change of each sample. With each original fold change value above the median fold change value, the fold change of the samples was summed up before dividing by the number of samples, leading to a penetrance fold change value. All proteins with negative frequency differential were filtered out. The output from the statistical analysis gives a list of highly significant antigens with high penetrance that may be related to *P. knowlesi* infection. Formulae used are stated below.

$$Overall\,fold\,change, O_{FC} = (mean\,RFU\,(case)/(mean\,RFU\,(control))$$

$$Frequency\,above\,median, f_{FC} = \sum_{i}^{n} x = median_i > FC_i$$

$$Penetrance\,fold\,change = f_{FC}/F$$

The penetrance fold change and the penetrance frequency for all proteins in the samples were used to rank the antigens results. The proteins were then filtered using a rule-based system (Table 2).

2.6. Functional analysis

The biological association between the selected proteins and the disease condition was assessed. The list of antigens with high reactivity was used to perform the functional analysis. Biological pathways related to the disease were obtained via PANTHER, a publicly available database. The gene ontology (GO) categories for the proteins were assigned using AmiGO 1.8. Besides, a text mining approach was used to acquire information on the antigens from literature.

Table 1
Age and sex matched healthy individuals and patients.

	Patient positive with knowlesi infection					Healthy individual with no malaria infection			
	Code/initial	Age	Gender	Race	Parasitemia	Code/initial	Age	Gender	Race
1	UM0002	32	Male	Malay	1.02%	SN3	33	Male	Malay
2	UM0015	36	Male	Malay	[a]	SN29	36	Male	Malay
3	UM0018	22	Male	Chinese	1.52%	SN101	23	Male	Chinese
4	UM0020	52	Male	Chinese	0.21%	SN37	52	Male	Chinese
5	UM0032	29	Male	Chinese	0.3%	SN71	29	Male	Chinese
6	UM0001	55	Male	Chinese	0.04%	SN15	54	Male	Chinese
7	UM0009	55	Male	Chinese	22.8%	UM0042	54	Male	Chinese
8	KH001	53	Male	Chinese	0.02%	SN51	53	Male	Chinese
9	AH001	29	Female	Malay	0.25%	SN109	28	Female	Malay
10	NA007	14	Female	Malay	0.01%	UM0052	19	Female	Malay
11	UM0029	34	Male	Sabahan	10%	SN135	34	Male	Sabahan

[a] Percentage of parasitemia not available.

Antigen with high reactivity
Difference in frequency must be at least 1
Frequency in control must be 0
The penetrance fold change in patient must be ≥ 1.1
The difference in penetrance fold change is >1

3. Results

3.1. Antigens with high reactivity with antibodies

Reactivity of IgG autoantibodies in the patient and control sera was analyzed using the OGT Discovery Array. The average within array variability for protein content, in terms of coefficient of variance (CV%) is 16.7% while the average CV% for Cy3-markers is 3.89%. Outliers were identified using principal component analysis (PCA) and removed during data QC. PCA was performed at the level of the arrays and the conclusion from the three scatter plots is that a large part of the data is clustered together (data not shown). After statistical analysis, a list of highly significant antigens with high penetrance fold change that may be related to *P. knowlesi* infection was generated. A high penetrance fold change would mean a higher reactivity of the antigens with antibodies. Using a rule-based system, 24 out of the 1636 array proteins, with the highest difference in frequency of antibodies and penetrance fold change between patient and control serum were identified (Table 3). We found all patient samples to have at least 1 AAb, except for 3 patient samples (KH001, UM0015 and AH001) which had none. Among the 8 patients with AAbs, 4 patient samples (UM0032, UM0002, UM0029 and UM0018) had more than 5 AAbs present in them.

3.2. Functional analysis

The 24 antigens listed (Table 3) were subjected to functional analysis. Using GO Slimmer of AmiGO ver. 1.8 [19], it was found that 22 antigens are intracellular while 5 of the antigens can be found in the extracellular region (Fig. 1B). The biological process analysis showed that the antigens are related to biosynthetic processes and cellular nitrogen compound metabolic processes (50%), signal transduction (45.8%), cellular protein modification process and response to stress (41.7%), cell proliferation (25%), cell death (20.8%), immune system process (25%) and others (Fig. 1A). The molecular functions (Fig. 1C) of the antigens were also analyzed with most antigens having binding activity and enzyme activity. Pathway analysis was done using Panther [20]. A total of 18 pathways were mapped (Table 4).

4. Discussion

In this study, we present for the first time an AAb profile of knowlesi-infected patients using OGT's patented protein array. The OGT Discovery Array consists of 1636 antigens which are involved in immune responses. Each protein is expressed in insect cells with a proprietary BCCP tag which is biotinylated post-translationally *in vivo* by the host cell. The ability of a BCCP fusion protein to become biotinylated depends on the correct folding of the protein. Thus, with BCCP acting as a folding marker, only correctly folded, functional native proteins are immobilized on the array. Each antigen on the array is printed in quadruplicate and the reproducibility of the platform has been reported to be high (CV <2.0% inter-assay after normalization) [1]. These features help ensure

Table 3
Proteins showing the highest difference in penetrance frequency of antibodies and penetrance fold change between the patients and controls.

Gene name	Name of protein and UniProt accession number	Penetrance fold change of patient	Penetrance fold change of control	Difference in penetrance fold change	Penetrance frequency among patient samples	Patient sample
SCAND1	SCAN domain-containing protein 1 (P57086)	9.83	2.17	7.66	1	UM0032
SPO11	Meiotic recombination protein SPO11 (Q9Y5K1)	7.64	1.81	5.83	1	NA007
FKBP3	Peptidyl-prolyl cis-trans isomerase FKBP3 (Q00688)	7.49	2.10	5.39	2	UM0002, UM0001
CITED1	Cbp/p300-interacting transactivator 1(Q99966)	6.38	2.65	3.73	2	UM0009, UM0029
CBFA2T3	Myeloid translocation gene 16 (O75081)	6.07	2.40	3.67	1	UM0002
GADD45G	Growth arrest and DNA damage-inducible protein GADD45 gamma (O95257)	5.74	2.00	3.74	1	UM0009
TTRAP	TRAF and TNF receptor-associated protein/Tyrosyl-DNA phophodiesterase 2 (O95551)	5.17	1.67	3.50	1	UM0029
NOL4	Nucleolar protein 4 (O94818)	5.00	1.91	3.09	3	UM0032, UM0020, UM0029
MAGEA4	Melanoma-associated antigen 4 (P43358)	4.80	1.81	3.00	2	UM0002, UM0029
BID	BH3-interacting domain death agonist (P55957)	4.14	2.16	1.98	1	UM0002
FGFR1OP	FGFR1 oncogene partner (O95684)	3.75	1.74	2.02	2	UM0032, UM0029
PSMD4	26S proteasome non-ATPase regulatory subunit 4 (P55036)	3.72	1.96	1.76	2	UM0009, UM0020
PPP3CA	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform (Q08209)	3.63	1.92	1.72	1	UM0032
PPP4R1	Serine/threonine-protein phosphatase 4 regulatory subunit 1 (Q8TF05)	3.57	1.29	2.28	1	UM0002
GPHN	Gephyrin (Q9NQX3)	3.54	1.75	1.79	3	UM0002, UM0029, UM0018
C9orf86	Rab-like protein 6 (Q3YEC7)	3.32	1.41	1.92	1	UM0032
ALOX15	Arachidonate 15-lipoxygenase (P16050)	3.26	1.5	1.77	1	UM0032
TPM1	Tropomyosin alpha-1 chain (P09493)	3.16	1.78	1.38	1	UM0029
BASP1	Brain acid soluble protein 1 (P80723)	3.02	1.56	1.46	3	UM0032, UM0029, UM0018
AK1	Adenylate kinase isoenzyme 1 (P00568)	2.92	1.36	1.56	2	UM0020, UM0029
SMAD2	Mothers against decapentaplegic homolog 2 (Q15796)	2.90	1.81	1.09	3	UM0002, UM0029, UM0018
SGK3	Serine/threonine-protein kinase Sgk3 (Q96BR1)	2.65	1.56	1.08	2	UM0029, UM0018
C1orf57	Cancer-related nucleoside-triphosphatase (Q9BSD7)	2.58	1.36	1.22	1	UM0032
MAP2K6	(P52564)	2.41	1.39	1.02	4	UM0032, UM0020, UM0029, UM0018
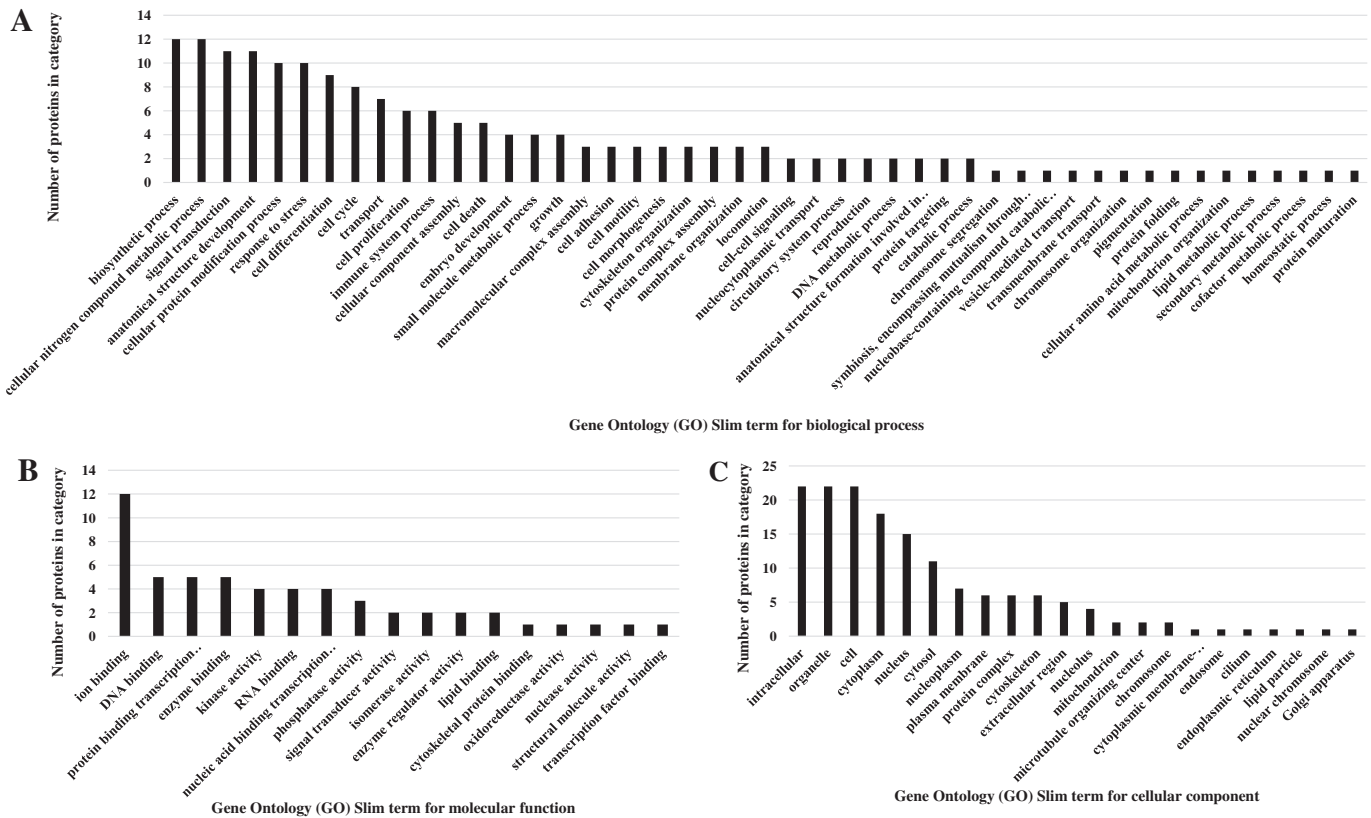
Fig. 1. Gene Ontology (GO) analysis of the 24 reactive antigens. (A) GO categorization for biological processes. (B) GO categorization for cellular components. (C) GO categorization for molecular functions.

that data from the protein array are reliable and meaningful. However, due to the nature of the proteins on the array, we were not able to elucidate tissue- or cell-specific AAbs such as those shown in the past which include, lymphocytes [7], erythrocytes [10] and smooth muscle [6,21]. Instead, it provided us with a molecular understanding of the host's immunological response towards the parasite, as majority of the autoantigens implicated are involved in various pathways related to the immunopathogenesis of the disease.

Table 4
Pathway analysis using PANTHER.

	Pathway accession	Pathway name	Mapped antigens
1	P00010	B cell activation	PPP3CA
2	P00053	T cell activation	PPP3CA
3	P06209	ALP23B signaling pathway	SMAD2
4	P00018	EGF receptor signaling pathway	MAP2K6
5	P00021	FGF signaling pathway	MAP2K6, PPP4R1
6	P06664	Gonadotropin-releasing hormone receptor pathway	MAP2K6, SMAD2, ALOX15, PPP3CA
7	P00052	TGF-beta signaling pathway	SMAD2, CITED1
8	P00031	Inflammation mediated by chemokine and cytokine signaling pathway	ALOX15
9	P06210	Activin beta signaling pathway	SMAD2
10	P00013	Cell cycle	PSMD4
11	P00057	Wnt signaling pathway	PPP3CA
12	P05918	p38 MAPK pathway	MAP2K6
13	P00059	p53 pathway	GADD45G
14	P06215	MYO signaling pathway	SMAD2
15	P04393	Ras pathway	MAP2K6
16	P00046	Oxidative stress response	MAP2K6
17	P02738	De novo purine biosynthesis	AK1
18	P00060	Ubiquitin proteasome pathway	PSMD4

AAbs were found in sera of 72% of individuals living in a malaria endemic area compared to only 17% observed in the normal control population [22]. Levels of IgG, IgM, IgA and tissue-reactive autoantibodies were also found to be significantly higher in Europeans who acquired malaria compared to healthy controls [21]. AAb formation during malaria infection cannot be explained by a generalized polyclonal B cell activation. Instead, it is a result of specific activation of autoreactive B lymphocytes [23,24]. It is believed that the simultaneous presence of immunogenic amounts of autoantigens together with parasite mitogens [25] would induce AAb formation during malaria infection. Autoantibodies may play an immunoprotective role [23,26]. Evidence has shown that sera and IgG antibodies from patients with systemic lupus erythematosus (SLE), an autoimmune disease, can inhibit the *in vitro* growth of *P. falciparum* [27,28]. Monoclonal AAb derived from transformed lymphocytes of mice was also able to inhibit *P. falciparum* growth, up to 40% [29].

Production and secretion of AAbs are regulated directly by the availability of respective antigens formed, as a homeostatic reaction [30]. Despite *Plasmodium* being a blood-borne parasite that invades erythrocytes which have no nuclear matter, we found that antigens with high reactivity towards their AAbs are mostly intracellular, with more than half found in the nucleus (Fig. 1B). This can be contributed by the apoptosis of mononuclear cells, lymphocytes or endothelial cells [31,32], a common feature in the pathogenesis of malaria. Apoptosis of these nucleated cells and erythrocytes releases intracellular self-antigens that stimulate the production of AAbs to curb the aberrant condition caused by the parasite. Wounding, apoptosis, or infection can trigger a proliferative response in neighboring cells to replace damaged cells [33]. Thus, pro-apoptotic (e.g., peptidyl-prolyl cis-trans isomerase FKBP3 and BH3-interacting domain death agonist BID) [34,35] and anti-apoptotic components, for example, TRAF and TNF receptor-associated protein (TTRAP) and Rab-like protein 6 (C9orf86) coexist at the same time at

a high level [36] in patients' sera. Besides, a high reactivity of cytoskeletal component AAbs (tropomyosin alpha-1 chain and gephyrin) was also found in this study. Mice experimentally infected with *P. chabaudi* develop a marked degree of polyclonal B cell activation and an intense immunological activity against cytoskeletal components such as actin, myosin, myoglobin, spectrin, and tubulin autoantigens [37]. Also, previous study by Bansal et al. [9] showed that circulating IgG from patients with cerebral malaria highly reacts with recombinant human beta tubulin III.

A delicate balance between pro- and anti-inflammatory responses exists in malaria [38]. The progression of chronic inflammatory processes such as that found in malaria, alters the damaged tissues, inducing the expression of new self-antigens recognized by natural AAbs which may be protective in nature [4]. Beneficial autoimmunity against proinflammatory cytokines has also been reported to play a role in the immunomodulation of autoimmune diseases [39]. Hence, antigens involved in pro- and anti-inflammatory processes could be activated or repressed at the same time during the pathogenesis of malaria. Consequently, the reactivity and availability of AAbs towards self-antigens vary accordingly. Here, it is found that there is a high reactivity between arachidonate 15-lipoxygenase (ALOX15) and its AAb. Upregulation of ALOX15 by IL-4 generates 15-hydroxyicosatetraenoic acid (15-HETE) [40], a potent activator of peroxisome proliferator-activated receptor gamma (PPARγ), and is responsible for many anti-inflammatory actions. However, 15-HETE was also found to stimulate red blood cells adherence to capillary endothelia, enhance vascular permeability and edema, and induce chemotaxis and chemokinesis [41], processes that could lead to severe malaria. Therefore, it is probable that AAb against ALOX15 is produced in the body to regulate both processes.

Based on the pathway analysis (Table 4), there were occurrences where multiple reactive AAbs, identified in individual patient samples are involved in familial pathways. In the patient sample UM0002, CITED1 and SMAD2 were identified. Both antigens participate in transforming growth factor-beta (TGF-β) signaling, mediated by the SMAD pathway [42]. CITED1 and SMAD2 form complexes with SMAD4 [43] to activate transcription following activation by TGF-β or activin. Transforming growth factor-beta downregulates production of pathogenic pro-inflammatory cytokines during the early stages of *P. falciparum* infection. This may facilitate parasite growth in the host and control inflammatory processes, leading to reduced immunopathology and preventing disease severity [44]. It was found that lower levels of TGF-β are associated with acute [45] and severe [46] falciparum malaria. Besides that, at least 2 of the 4 antigens involved in the gonadotropin-releasing hormone (GnRH) receptor pathway, were found in patient sample UM0032, UM0029 and UM0018. Additionally, antigens associated with the p38 MAPK pathway were identified in patient sample UM0032. The antigens ALOX15 (through the product 15-HETE) and MAP2K6 promotes the phosphorylation of p38 MAPK [47, 48]. These AAbs identified together within individual patient samples, may play a role during knowlesi malaria progression through the familial pathways they regulate.

The 24 reactive antigens shown in this study are involved in inflammatory processes in the host. It is believed that less specific markers, such as those that indicate inflammatory status and oxidative stress can predict disease severity in malaria [49]. On the other hand, studies have also shown that asymptomatic malaria can be highly prevalent [15,50] and not easily detected. Autoantibodies are abundant in the serum, easily purified and detected and their responses are specific [18]. In certain diseases such as rheumatoid arthritis, cancer and SLE, AAbs can even be detected before the onset of clinical symptoms [1]. With further research and validation, AAbs may have the potential to serve as predictive biomarkers for disease severity in malaria and for cases of asymptomatic malaria or mild malaria to monitor malaria reservoirs.

Although the findings indicate host AAb–autoantigen interactions, the existence of cross-reactions of antibodies against parasite antigens with self-antigens should be considered. It is obvious that molecular mimicry between malaria parasites and various self-antigens exists [51–53]. Similarities between the host antigens and parasite antigens could exist, and this notion of antigen sharing between parasites and their hosts has been demonstrated [54]. Humans and *Plasmodium* do possess certain molecules with sequence homology such as the kinases (eg. MAPK), phosphatases (eg. PP2A) and immunophilin of the FKBP family (eg. FKBP3) [55–57]. Thus, it is difficult to find the original immunogenic stimulus as the AAbs elucidated could initially be antibodies against the parasite, not self, and vice versa.

This study has provided us a "snapshot" of the patients' autoantibody profiles during *P. knowlesi* infection. The findings give an insight of the molecular immunopathogenesis of the disease, especially from an autoimmunity point of view. Due to limited number of patient sera available, the sample size of this study is relatively small. Moreover, there were 3 patient samples (KH001, UM0015 and AH001) which did not exhibit any reactive AAb while other samples had variable number of reactive AAbs. This may be caused by heterogeneity of the patients and the disease leading to an imbalanced pattern of AAb reactivity within the group of samples. A larger sample size could be employed in the future to consolidate and further characterize these findings. Patients with falciparum and vivax malaria could also be included to determine the specific autoimmune response towards knowlesi malaria. Future work can also be directed to measure the level of the autoantigens as there has been observation that certain proteins were strongly recognized by IgG although present in low amounts whereas others were weakly or not recognized although present in high amounts [3]. Nonetheless, with this information regarding the coexistence of autoimmunity and malaria, it would be helpful to consider the development of autoimmunity in the design of immunoprophylactic measures so that protection of humans against malaria can be effective.

Acknowledgments

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