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Elevated plasma α1-antichymotrypsin is a biomarker candidate for malaria patients

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Advancements in the field of proteomics have provided opportunities to develop diagnostic and therapeutic strategies against various diseases. About half of the world's population remains at risk of malaria. Caused by protozoan parasites of the genus *Plasmodium*, malaria is one of the oldest and largest risk factors responsible for the global burden of infectious diseases with an estimated 3.2 billion persons at risk of infection. For epidemiological surveillance and appropriate treatment of individuals infected with *Plasmodium* spp., timely detection is critical. In this study, we used combinations of depletion of abundant plasma proteins, 2-dimensional gel electrophoresis (2-DE), image analysis, LC-MS/MS and western blot analysis on the plasma of healthy donors (100 individuals) and vivax and falciparum malaria patients (100 vivax malaria patients and 8 falciparum malaria patients). These analyses revealed that α1-antichymotrypsin (AACT) protein levels were elevated in vivax malaria patient plasma samples (mean fold-change ± standard error: 2.83 ± 0.11, based on band intensities), but not in plasma from patients with other mosquito-borne infectious diseases. The results of AACT immunoblot analyses showed that AACT protein was significantly elevated in vivax and falciparum malaria patient plasma samples (≥ 2-fold) compared to healthy control donor plasma samples, which has not been previously reported. [BMB Reports 2022; 55(11): 571-576]

INTRODUCTION

Malaria is a significant public health burden with more than 3.2 billion people being at risk of infection, especially in the resource-poor settings (1). In 2020, 241 million malaria cases and 627,000 deaths were reported globally. In humans, malaria is exclusively caused by infection with a *Plasmodium* species (*Plasmodium falciparum*, *P. vivax*, *P. kwowlesi*, *P. ovale*, or *P. malaria*) that is transmitted to humans through the bite of infected female *Anopheles* mosquitoes (1-3). Since the re-emergence of infectious vivax malaria in 1993, Republic of Korea (Korea) has made significant progress in eliminating vivax malaria and has been consequently categorized as one of 32 malaria-eliminating countries (4). Despite enormous control efforts, mortality and morbidity caused by malaria remain high in many developing countries (5-8). Malaria is a treatable and preventable condition (9).

Prompt and accurate diagnosis of malaria alongside reliable identification of *Plasmodium* species is pivotal for rapid and effective disease management, as a late diagnosis can result in significant morbidity and mortality. For prompt an accurate diagnosis and treatment of malaria, more sensitive diagnostic tools should be provided to detect asymptomatic and sub-microscopic infections that contribute to transmission (10). Accurate diagnosis and appropriate treatment of malaria are the keys to global malaria elimination, but the lack of accurate diagnostic tools has led to poor prognosis and delayed treatment (11). Recently, omics-driven technologies have represented as an advancement in diagnostics and cell-based diagnosis, immune-chromatographic tests for parasitic proteins including *p*HRPII (*Plasmodium* histidine rich protein II), *p*LDH (*Plasmodium* lactate dehydrogenase) and aldolase, and mass-spectrometry based proteomics for host and parasite proteins (12, 13). Omics encompasses robust technique for approaching a biological problem from varying points of reference (14). However, multi-omics or integrated omics remains a challenge in malaria detection, therefore optical microscopy still remains the gold standard technology for malaria diagnosis in most parts of the malaria-endemic world (14, 15).

Traditional proteomic accesses can elucidate protein expression profiles, which may have promising applications to clinical cases, therapeutic responses, or investigation of the elucidating mechanisms of diseases such as cardiovascular diseases, autoimmune disorders, and various cancers (16, 17). Biomedical and clinical proteome research targeted at biomarker discovery is majorly founded on expression proteomics, which investigates

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the quantity of specific proteins in different conditions. Thus, proteomic studies are likely to be pivotal factors in accelerating the manifestation of new biomarkers.

Glycoprotein α1-antichymotrypsin (AACT) is a protein ascribed to the serine proteinase inhibitors superfamily, also known as serpins (18). AACT is involved in, inflammation, proteolysis, and the acute phase response. Although AACT is mainly synthesized in the liver, after which it is secreted into the blood, it is also synthesized in the brain, mainly by astrocytes (19, 20). In the brains of patients suffering from Alzheimer's disease, AACT has been reported to bind amyloid-β peptides found in plaque cores and blood vessels and the protein is overexpressed in the brain of Alzheimer's disease patients (21, 22). Its expression is controlled by proinflammatory cytokines including oncostatin M, interleukin (IL)-1, and complexes of IL-6, soluble IL-6 receptors, and transcriptional regulators such as activator protein 1 and nuclear factor 1-X (23). However, AACT's potential as a biomarker for Alzheimer's disease is controversial. These proteins inactivate proteinases with a serine residue in their active site (24). AACT is a major acute-phase reactant, and its concentration in plasma increases in response to trauma, surgery, and infection and its elevated level is widespread, but not universal (25, 26). The dysregulation of AACT expression and its glycosylation states are linked with tumor progression and recurrence and could be exploited as a biomarker for tracing tumors, including in diffuse large B-cell lymphoma, pancreatic cancer, liver cancer, ovarian cancer, and lung cancer (27). However, the expression changes in protein level, biological functions, and glycan modification of AACT is to be elusive.

With this purpose, in this study, we initially studied plasma proteins of patients suffering from vivax malaria alongside those of healthy specimen to deduce biomarkers for the discrimination between long-term and short-term latent malaria. We also sought to acquire a thorough elucidation of the pathophysiological mechanism of the infectious disease by utilizing confluences of depletion of abundant proteins, 2-dimensional gel electrophoresis (2-DE), gel-image analysis, and exact mass spectrometry. Though it failed to discriminate between short-term and long-term latent malaria, a plasma glycoprotein, AACT could be developed as a possible biomarker for *Plasmodium* malaria, but not for other mosquito-borne infectious diseases.

RESULTS

To identify new serologic biomarker candidates for malaria, we used plasma samples acquired from healthy donors and patients confirmed to be infected with vivax malaria. An ordinary 2-DE pattern of plasma from which 14 highly abundant proteins were removed as described previously is shown in Supplementary Fig. 1A. Although the primary goal of this study was to discover biomarkers to discriminate between short-term (S group) and long-term (L group) latent malaria, no differentially expressed proteins could be found in this study that would distinguish these two latent types of malaria. We leaved out those proteins that are previously known or involved in other diseases as well as those for which commercial antibodies are not available. Thus, AACT which was overexpressed in malaria plasma samples (2.5-fold in the L group compared with the control group, 2.6-fold in the S group compared with the control) was chosen as the sole candidate protein (Supplementary Fig. 1B). To verify AACT as a vivax malaria biomarker candidate and to assess its efficiency and specificity in diagnosing vivax malaria, we used 200 individual plasma samples from healthy donors (n = 100)

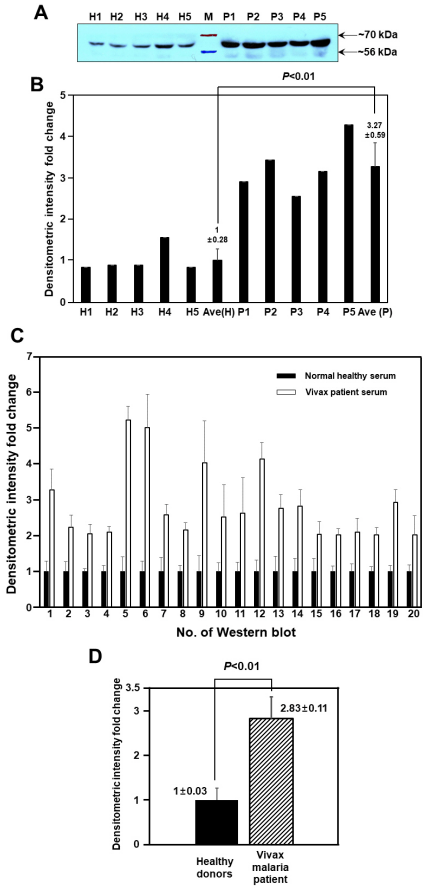

Fig. 1. (A) AACT in the plasma of healthy donors and vivax malaria patients detected by chemiluminescence (Blot #1): H numbered lanes are healthy group (1-5) and P numbered lanes (1-5) are patients. (B) Expression level of AACT in specimens was measured by comparing the band intensities of malaria-patient samples with those of healthy donors. Data in Ave. lanes are presented as means ± standard errors of five independent lanes. AACT expression differed significantly between plasma from healthy donors (#H1-#H5) and plasma from donors with vivax malaria (#P1-#P5; $P < 0.01$). (C) Expression level of AACT in specimens was measured by comparing the band intensities of each patient sample with that of the healthy donors (Blot #1-#20). (D) $P < 0.01$, compared with healthy donor (#H1-#H100) versus vivax malaria patients (#P1-#P100).

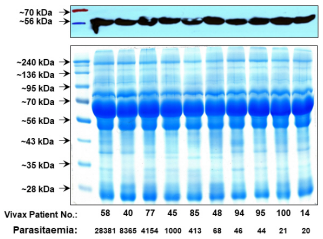

Fig. 2. Western blot analysis and SDS-PAGE of the plasma samples from individuals with parasitaemia and individuals infected with *P. falciparum*. Equal amount of whole plasma from patients with different severities of parasitaemia was subjected to Western blot analysis and SDS-PAGE.

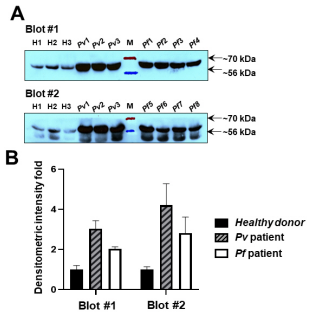

Fig. 3. Western blot analysis and SDS-PAGE of the whole plasma from falciparum malaria patients. (A) Samples from three vivax malaria patient (*Pv*1-*Pv*3) and three healthy donor (H1-H3) were used for positive and negative controls. Blot #1 represents 4 falciparum malaria patients (*Pf*1-*Pf*4) and blot #2 represents *Pf*5-*Pf*8. (B) Expression level of AACT in specimens was measured by comparing the band intensities of each falciparum malaria patient sample with those of healthy donors as negative control and those of vivax malaria patients as positive control: blot #1 represents *Pf*1-*Pf*4 and blot #2 represents *Pf*5-*Pf*8.

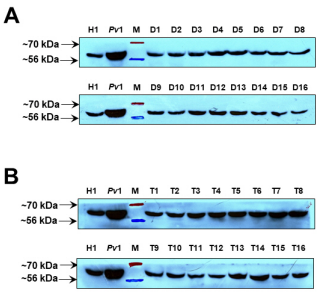

Fig. 4. Western blot analysis and SDS-PAGE of whole plasma from other mosquito-borne disease with healthy donor (H1) and vivax malaria patient (Pv1) samples. (A) Western blot analysis and SDS-PAGE of Dengue fever patients (D1-D16). (B) Western blot analysis and SDS-PAGE of Tsutsugamushi disease patient samples (T1-T16).

and vivax malaria patients (n = 100); specifically, we evaluated differential AACT expression Western blot analysis, using a specific monoclonal antibody against AACT. As shown in Fig. 1, and Supplementary Fig. 2, the protein expression level of AACT in vivax malaria-patient samples was more than 2-fold higher than that in the control (means ± standard errors, Control: 1 ± 0.03 and vivax malaria patient: 2.83 ± 0.11, based on band intensities, Fig. 1D). These results indicates that AACT is highly expressed in the plasma of vivax malaria patients. Loading (30 μg) of each sample on the gel was normalized by staining with a Coomassie brilliant blue G250 (CBB G250) staining solution. Malaria is transmitted by female *Anopheles* species mosquitoes and results in peripheral blood parasitaemia which may manifest asymptomatically in adults residing in malaria occuring areas (28). The patients whose plasma was used for Western blot analysis had parasitaemia with between 20 and 28,381 parasites/μl (means ± standard deviations, 3,504.9 ± 6,159.1 parasites/μl). It is well-established that optical microscopical screening is able to identify parasite species and establ;ish parasite densities. However, the limitation of detection by optical microscopy technology is in the range of 5 to 100 parasites/ml of blood (29, 30). It also requires experienced technicians and a microscope, which are not always available in remote areas. Despite differences in their parasitaemia of vivax patients, the AACT band intensities produced from Western blot analysis did not differ according to the severity of parasitaemia (Fig. 2). According to the Korea Disease Control and Prevention Agency, *P. falciparum* infections occurring in Korea were imported rather than domestic cases (31, 32). In this study, eight imported falciparum malaria-patient plasma samples were also subjected to Western blot analysis with the anti-AACT antibody. The protein expression level of AACT in falciparum malaria samples was more than 2-fold higher than that in control samples, indicating that AACT is also highly expressed in the plasma of falciparum malaria patients (Fig. 3). In practice, there is no evidence-based information to lead clinicians in giving presumptive medical treatment when malaria is strongly predicted, and laboratory confirmation is not immediately available (33).

Next, to elucidate the relative expression level of AACT in other mosquito-borne infectious diseases, we evaluated the comparative diagnostic specificity of AACT for malaria and other mosquito-borne infectious diseases including Tsutsugamushi disease and Dengue fever. We performed Western blot analyses using16 Dengue fever patient plasma samples (Fig. 4A) and 16 Tsutsugamushi disease patient plasma samples (Fig. 4B). As shown in Fig. 4, the expression pattern and level of AACT in Tsutsugamushi disease and Dengue fever samples differed from that of vivax and falciparum malaria plasma and was similar to that of healthy plasma.

DISCUSSION

Our results might confirm the malaria-specific up-expression of AACT in vivax and falciparum malaria. Malaria is presupposed

primarily on the base of fever or a history of fever (4). There is no combination of symptoms or signs that reliably discriminates malaria from other causes of fever, and diagnosis relied only on clinical symptoms has very low specificity and results in bad treatment (9). To guide rational use of antimalaria medicines, the focus of malaria diagnoses should be to discriminate patients who truly suffer from malaria. The two practical tools used routinely for parasitological diagnosis of malaria are optical microscopy, the gold standard diagnosis for malaria, and immunochromatographic rapid diagnostic tests (RDTs). Optical microscopy of stained blood smears has important advantages including low direct cost, high sensitivity, differentiation of *Plasmodium* species, and determination of parasite densities. However, the accuracy of microscopy examination can be awkward to maintain because of the need to train and supervise of laboratory staff. On the other hand, using a finger-prick blood sample, RDTs detect parasite-specific antigens based on the detection of HRP2, species-specific or pan-specific Plasmodium LDH or pan-specific aldolase (2). Although these tests have advantages, including rapid provision, fewer requirements for training personnel, and reinforcement of patient confidence, they also have many potential disadvantages; these disadvantages include the panel detection score of at least 75% at 200 parasites/μl, the false positive rate, the existence of lot-to-lot variation, the frequencies of HRP2 deletions, and the poor sensitivity for detecting *P. ovale* and *P. malariae*. In this study, the expression level of AACT in either vivax malaria or falciparum malaria-patient plasma was upregulated. This analysis involved 100 vivax malaria blood samples and 8 falciparum malaria blood samples. Microscopy examination and RDTs are to seek sensitivity for low-level parasitaemia. The lower limit of detection (LOD) for microscopy is between 50 and 500 parasites/μl relying on the microscopist's expertise (34, 35) and 100 parasites/μl for *Pf*HRP2 (*P. falciparum* histidine rich protein 2) based *P. falciparum* RDTs (36). Recently, a multiplex qPCR analysis was developed that has high sensitivity and high negative predictive value even in the LOD range of 0.05 parasites/μl including all *Plasmodium* species was developed (37). However, this assay does not resemble a field deployable rapid test due to its expense and the requirement of advanced laboratory infrastructure. Here, using proteomics-based technology, we identified a novel glycoprotein biomarker candidate even in samples reflecting low-level parasitaemia (20 parasites/μl in blood) at the expression level of 2.83 ± 0.11 fold, based on Western blot analysis band intensities. The test for AACT was not responsive to other mosquito-borne parasitic infectious diseases including Tsutsugamushi disease and Dengue fever.

Although this study demonstrated that AACT could serve as a novel biomarker for malaria caused by *P. vivax* and *P. falciparum*, there are some limitations. First, this study is a retrospectively designed study showing the potential usefulness of preoperative plasma AACT to predict prognosis by using small and heterogenous samples. To acquire more statistical power, a large sample size including other types of malaria such as, *P. falciparum* as well as different life cycle stages (trophozoite, ring stage, gametocyte, and schizont) is necessary in future work. In addition, to qualify AACT as a novel biomarker for malaria, further studies of AACT's applications, such as its usefulness in detecting recurrence and monitoring treatment are required. In addition, because malaria is able to be transmitted by blood transmission between humans and is undoubtedly applicable to the majority of the world's transfusion transmitted diseases (38). For these reasons, and because the availability of molecular diagnostic tools is still limited, AACT might be used as a novel biomarker candidate to obtain information about low parasitaemia malaria and to diagnose *P. vivax* and *P. falciparum* infections.

In conclusion, we evaluated discriminatorily expressed proteins in the plasma of vivax malaria patients using traditional proteomic tools, which showed us to identify changes in AACT protein levels. Although the changes in AACT are likely not specific to vivax malaria patients, our study proposed the methodological achievements for a proteomic approach to examine plasma proteins in malaria patients. Additional in-depth investigation into the cellular and biochemical mechanism of AACT in malaria infection is warranted. These results have clinical implications in terms of the elucidation of *Plasmodium* spp. infection and improved diagnosis of malaria. These promising results suggest the potential of utilizing AACT as a biomarker to detect *Plasmodium* spp. including *P. vivax* and *P. falciparum* in blood samples.

MATERIALS AND METHODS

Ethics statement and sample collection

This study was performed under the regulation of the Institutional Review Board Committee of Konkuk University (No. 7001355-202007-BR-386). This research adhered to the tenets of the Declaration of Helsinki. The malaria patients' and healthy donors' plasma was obtained from the Global Resource Bank of Parasitic Protozoa Pathogens in Incheon National University. The vivax malaria patients' plasma was obtained from 100 Korean patients and the falciparum malaria patients' plasmas were from 8 Korean patients confirmed in the Inha University Hospital and Inha University Department of Tropical Medicine. The *Plasmodium*-negative blood samples were obtained from 100 healthy people from three administrative districts in Korea (Cheolwon, Hampyeong, and Bosung; randomized and blindly collected). Plasma from patients with Tsutsugamushi disease and Dengue fever confirmed in Chosun University and Inha University was also collected from the Global Resource Bank of Parasitic Protozoa Pathogens.

Sample preparation for proteomic analysis and protein identification

Originally, we categorized the plasma samples into three groups: i) healthy donor (C group), ii) patients for whom the outbreak time coincides with the mosquito activity period (S group), and iii) patients for whom the outbreak time does not coincide with

the mosquito activity period (L group). To remove 14 highly abundant proteins (albumin, immunoglobulin G (IgG), IgM, IgA, α1-antitrypsin, transferrin, haptoglobin, α2-macroglobulin, fibrinogen, complement C3, α1-acid glycoprotein (orosomucoid), HDL (apolipoproteins A-I and A-II), and LDL (mainly apolipoprotein B)) from human plasma, the multiple affinity removal column system based on avian antibody-antigen interactions (Seppro® IgY14, Millipore Sigma, St. Louis, MO, USA) was routinely used according to the manufacturer's recommended protocols. To search for a novel serologic indicator candidate for malaria, we carried out an integrated proteomic analysis using pooled plasma from healthy donors (C group) and vivax malaria patient groups (S and L groups) (39, 40). All procedures for the proteomic analysis, including 2-DE, image analysis, trypsin digestion, protein identification by LC-MS/MS, and data searches for protein identification were performed by Yonsei Proteome Research Center (Seoul, Korea) as previously described (39, 40).

Validation of the target proteins

Validation of some differentially expressed protein candidates was performed by Western blot analysis with the commercially available specific antibody. Total protein concentrations of plasma samples were estimated using a bicinchoninic acid-based protein assay system (Pierce, Rockford, IL, USA). Immunoreactive proteins on the membrane were detected using ECL Plus Western blotting detection reagents (GeneCure, Norcross, GA, USA). To evaluate band intensities, bands on the X-ray-films were imaged and analyzed using the ChemiDoc™ XRS + System equipped with Image Lab SoftwareTM (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data were expressed as means ± standard errors and analyzed by a Student's t-test. Statistical significance was accepted at $P < 0.01$. IBM SPSS Statistics ver. 27 (IBM, Somers, NY, USA) was used for all of the statistical analyses.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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