

## Original Article

# Increased expression of kidney injury molecule-1 and matrix metalloproteinase-3 in severe *Plasmodium falciparum* malaria with acute kidney injury

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**Abstract:** Kidney injury molecule-1 (KIM-1) is a specific histological biomarker for diagnosing early acute kidney injury which is markedly up-regulated in the proximal tubular cells in acute and chronic tubular injury. KIM-1 expression is poorly defined in the renal tubules of malaria patients. This present study aimed to determine KIM-1 expression as a specific biomarker for acute tubular damage and to identify matrix metalloproteinase-3 (MMP-3) as a mediator for KIM-1 shedding. Paraffin-embedded kidney tissues from autopsies of malaria patients were obtained from the Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Thailand. The kidney tissues were divided into 2 groups: those with acute kidney injury (AKI) (n = 10 cases) and those with non-AKI (n = 10 cases). Ten normal kidney tissues were used as a control group. The expression of KIM-1 and MMP-3 was examined by immunohistochemical staining. KIM-1 and MMP-3 expressions were strongly expressed in the proximal tubular cells in all kidney tissues from severe *Plasmodium falciparum* malaria with histological changes showing acute tubular damage. The mean intensity and total score of KIM-1 and MMP-3 expressions were significantly increased in proximal tubules of AKI group compared to non-AKI and control groups (all  $P < 0.001$ ). There was a significant positive correlation of total score of KIM-1 expression and the parameters of kidney function for AKI, including serum creatinine (Cr) and blood urea nitrogen (BUN). In addition, strong positive correlations were found between total score of KIM-1 expression and proximal tubular necrosis and MMP-3 expression. The study supports the potential role of KIM-1 as a specific biomarker for renal proximal tubular damage in malarial AKI, and indicates that the process of KIM-1 shedding might be stimulated by MMP-3.

**Keywords:** Kidney injury molecule-1, KIM-1, matrix metalloproteinase-3, MMP-3, acute kidney injury, AKI, malaria, *Plasmodium falciparum*

## Introduction

In an endemic area, acute kidney injury (AKI) is one of the most serious complications of severe *Plasmodium falciparum* malaria, occurring up to 40% of adult patients and is associated with high mortality rate [1-3]. AKI is also frequently reported in imported travelers with *P. falciparum* infection [4]. Currently, precise mechanism and pathogenesis of AKI in *P. falciparum* infection is not clearly known. The hypotheses of mechanical obstruction by infected erythrocytes, immune mediated glomerular and tubular pathology, fluid loss due to multiple mechanisms and alterations in the

renal microcirculation have been proposed [5, 6]. It has been reported that a major contributor for malaria AKI is caused by restricted local blood flow [6]. The main pathological findings of AKI in *P. falciparum* infection are glomerular proliferation, basement membrane thickening, presence of infected erythrocytes, hemosiderin and granular deposits, presence of hemoglobin casts in the tubular lumen, cloudy swelling of tubular cells, thinning of the tubular epithelium, interstitial edema, cellular casts, flattening of the renal tubular cells due to tubular dilation, patchy or diffuse denudation of the renal tubular cells and cellular necrosis [5-7]. The process of apoptosis was also reported in the tubular

cells of AKI with severe *falciparum* malaria and is correlated with acute tubular necrosis (ATN) [7]. Generally, the diagnosis of AKI depends on the alteration of serum creatinine (Cr) and blood urea nitrogen (BUN) levels, but both parameters are insensitive and non-specific for detection of renal injury [8, 9]. Currently, many researches attempt to discovery the reliable biomarkers for renal tubular injury to detect early tubular injury and evaluate the effectiveness of the new biomarkers.

Kidney injury molecule-1 (KIM-1) is a type 1 transmembrane glycoprotein which is a biomarker for renal proximal tubular damage [10]. KIM-1 is undetectable in normal kidney, but this protein is expressed in the apical surface of surviving proximal tubule epithelial cells after post-ischemia and ATN [11, 12]. During renal tubular injury, KIM-1 extracellular domain is cleaved by metalloproteinase and can be quantified in the urine as a biomarker for ATN [10, 13, 14]. A mouse model study of AKI induced by ischemia and reperfusion revealed an increased in KIM-1 and matrix metalloproteinase-3 (MMP-3) expressions, as well as their co-localization in kidney tissues from ischemic but not in sham-operated mice [15]. Previous report shows that urinary KIM-1 concentrations was elevated in malaria patients with AKI and proves that KIM-1 is a good diagnostic performance for AKI in *P. falciparum* malaria [16]. However, no study has reported the protein expression of KIM-1 and MMP-3 in AKI with severe *P. falciparum* malaria. To test the utility of KIM-1 as a specific marker for acute tubular damage in malaria patients with AKI and to identify MMP-3 as a sheddase for KIM-1 shedding in renal tubular cells, kidney tissues from autopsies of *P. falciparum* malaria patients were examined for the expression of the KIM-1 and MMP-3 by immunohistochemical staining. The correlations of KIM-1 with MMP-3 expression and clinical data were analyzed.

## Materials and methods

### Tissue specimens

Kidney tissues from fatal *P. falciparum* cases were classified into two groups: (1) *P. falciparum* with acute kidney injury (AKI) (Cr  $\geq$  265  $\mu$ mol/L or 3 mg/dl), and (2) *P. falciparum* without AKI (Cr < 265  $\mu$ mol/L), based on Cr level before death. Control kidney tissues were obtained from stored specimens collected from

accident fatalities with no kidney pathology, showing normal glomeruli, basement membrane, and tubules. The study population, clinical data and results of histopathological findings of renal tubules have been described in our previous report [7]. The use of leftover kidney specimens and the study protocol were reviewed and approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2016-059-01) and the Ethics Committee on Human Rights Related to Research Involving Human Subjects, Walailak University, Thailand (Protocol no. 15/011).

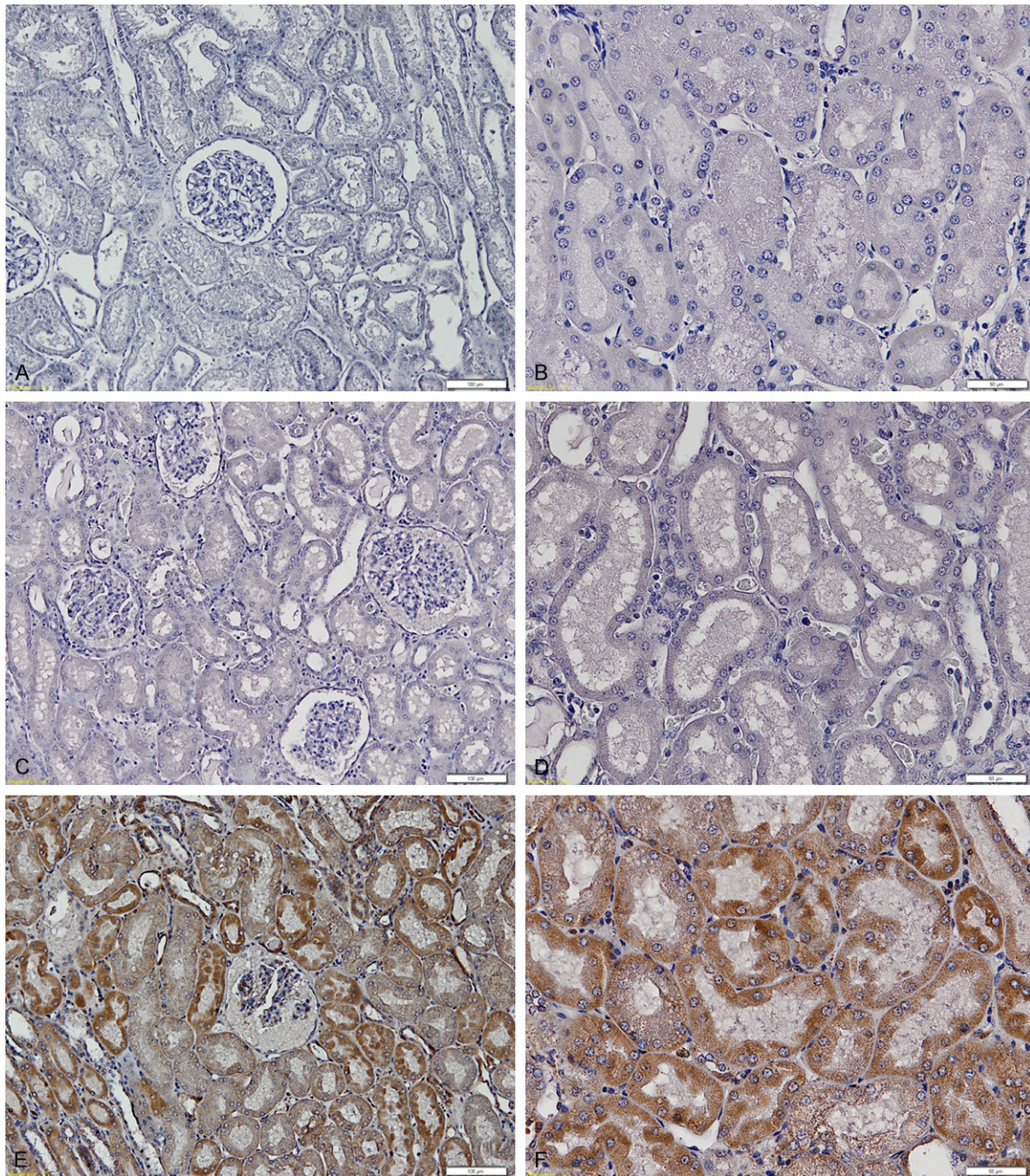
### Immunohistochemical study of KIM-1 and MMP-3

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue using the Vectastain ABC Kit (Rabbit IgG) (PK-6101, Vector Laboratories, Burlingame, CA). Sections were heated in an oven at 60°C for 30 min, deparaffinized in xylene and followed by rehydration through a descending ethanol series. Antigen retrieval was accomplished by microwave heating of tissue sections at 95°C for 20 minutes in citrate buffer (Vector Laboratories, Burlingame, CA). After cooling to room temperature, the slides were incubated with 3% hydrogen peroxide for 5 min to inhibit endogenous peroxidase activity and blocked for 30 min in normal goat serum. Anti-TIM-1/KIM-1 rabbit polyclonal antibody (1:100, Abcam, Cambridge, UK) or anti-MMP-3 rabbit monoclonal antibody (1:50, Abcam, Cambridge, UK) was incubated overnight at 4°C and then reacted with biotinylated secondary antibody for 30 minutes (Vector Laboratories, Burlingame, CA). To detect the bound rabbit antibody, the section was incubated with an avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Inc., Burlingame, CA) for 30 min, and with 3,3-diaminobenzidine to detect the peroxidase activity (brown staining). All steps included washing with phosphate buffered saline, pH 7.4. Slides were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany) and placed in permanent non-aqueous medium (Vector Laboratories, Burlingame, CA, USA).

### Evaluation of immunohistochemical staining

KIM-1 positive staining was defined as brown granular staining in the proximal tubules. Protein expressions of KIM-1 and MMP-3 were



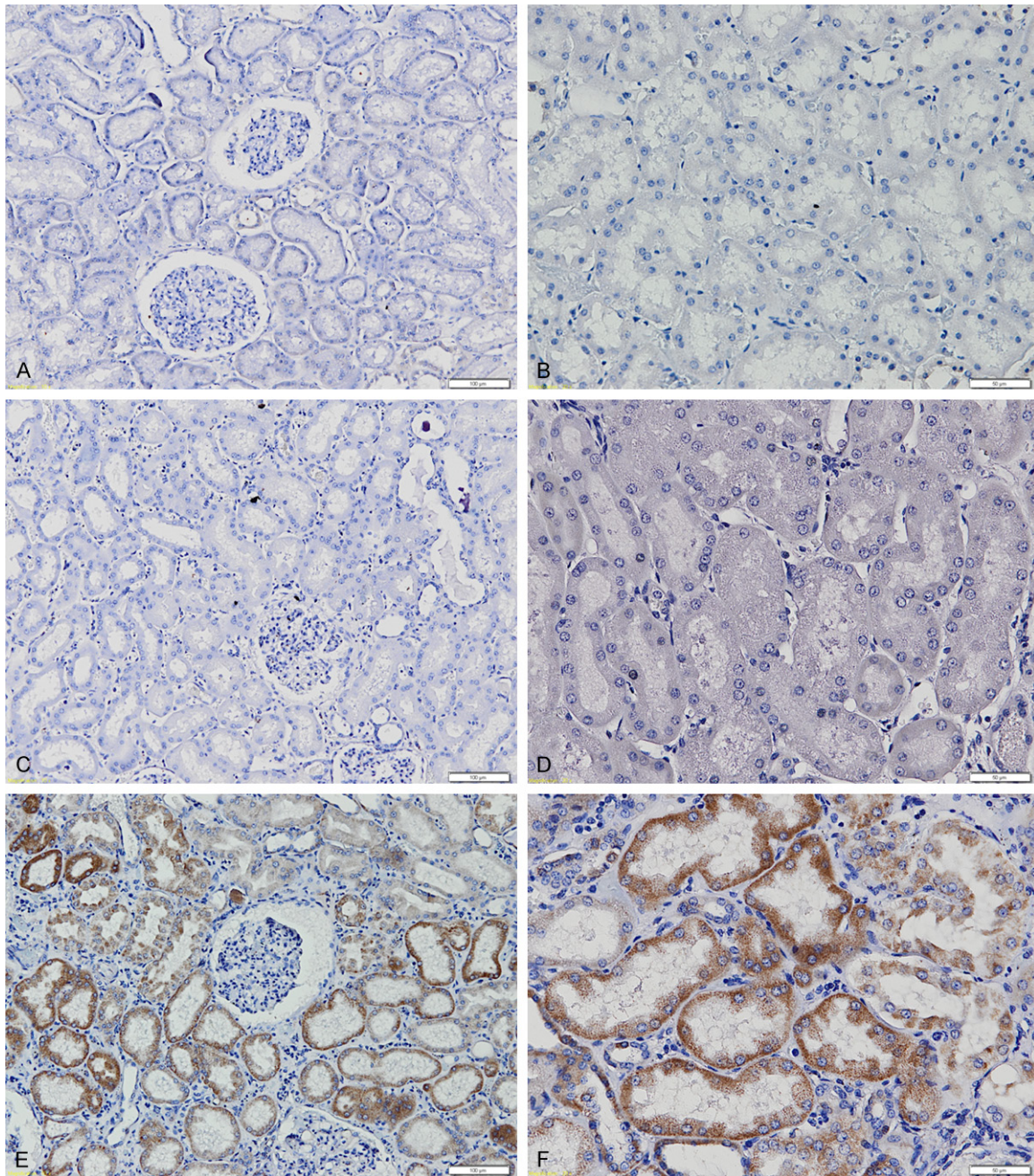


**Figure 1.** Expression of KIM-1 in kidney tissues. Normal kidney tissues showing negative staining for KIM-1 (A and B), kidney tissues from *P. falciparum* patients without AKI (non-AKI) (C and D), and kidney tissues from *P. falciparum* patients with AKI showing strong KIM-1 expression (E and F) (Original magnification  $\times 100$  in (A), (C), (E) and  $\times 200$  in (B), (D), (F)).

assessed using a grading system based on tubular epithelial cells with positive staining in cytoplasm or cellular membrane. KIM-1 and MMP-3 positive tubules were scored semi-quantitatively by estimating the percentage of tubules expressing KIM-1 or MMP-3 per field, which were divided into 4 categories based on

the staining criteria as follows: 0 = non-stained epithelial tubular cells or up to  $< 10\%$  of tubular epithelial cells with positive staining, 1 =  $10.1\%$  to  $25\%$  of tubular epithelial cells with positive staining, 2 =  $25.1\%$  to  $50\%$  of tubular epithelial cells with positive staining, 3 =  $50.1\%$  to  $75\%$  of tubular epithelial cells with positive staining





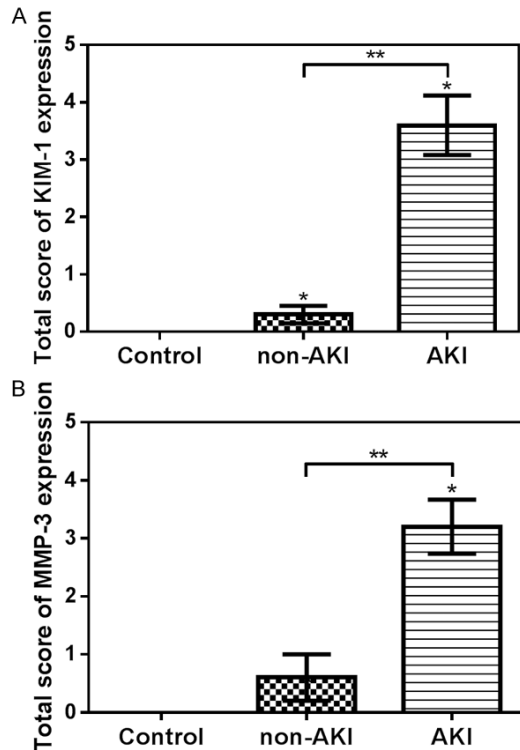
**Figure 2.** Expression of MMP-3 in kidney tissues. Normal kidney tissues showing negative staining for MMP-3 (A and B), kidney tissues from *P. falciparum* patients without AKI (non-AKI) (C and D), and kidney tissues from *P. falciparum* patients with AKI showing strong MMP-3 expression (E and F) (Original magnification  $\times 100$  in (A), (C), (E) and  $\times 200$  in (B), (D), (F)).

and 4 = 75.1% to 100% of tubular epithelial cells with positive staining, according to previous studies with minor modification [17]. In addition, staining intensity score for KIM-1 and MMP-3 was graded from 0 to 3. Finally, the total scores for KIM-1 and MMP-3 expression was calculated from the product of the percentage of positive tubules and intensity score [Total score = score for percentage of positive

tubules  $\times$  intensity score (I)]. All slides were examined by two independent observers using a double-blind review method.

## Statistical analysis

Data were analyzed using descriptive statistics. All quantitative data were presented as mean  $\pm$  standard error of mean (SEM). Normal distribu-



**Figure 3.** Semi-quantitative analysis for KIM-1 and MMP-3 expressions in the kidney tissues of control, non-AKI and AKI groups. \*Significance of  $P < 0.001$  compared with the control group. \*\*Significance of  $P < 0.001$  compared with non-AKI. Data are presented as mean  $\pm$  SEM.

tion of the variables was tested by Kolmogorov-Smirnov test. Differences in parameters among groups were analyzed by ANOVA followed by a post hoc test for multiple comparisons. The correlations between the KIM-1 and MMP-3 expressions and clinical data were calculated using the Spearman's correlation test. Statistical analysis was performed using SPSS version 17.0 software (SPSS, Chicago, IL). Statistical significance was set at  $P$ -value  $\leq 0.05$ .

## Results

### KIM-1 and MMP-3 expressions

Using immunohistochemical staining, KIM-1 positive staining was characteristically localized in the entire cytoplasm of the proximal tubular cells and extended into the lateral cellular membrane when epithelial junction appeared disrupted in area of tubular necrosis or damage. KIM-1 expression was not apparent in the apical membrane. The representative staining

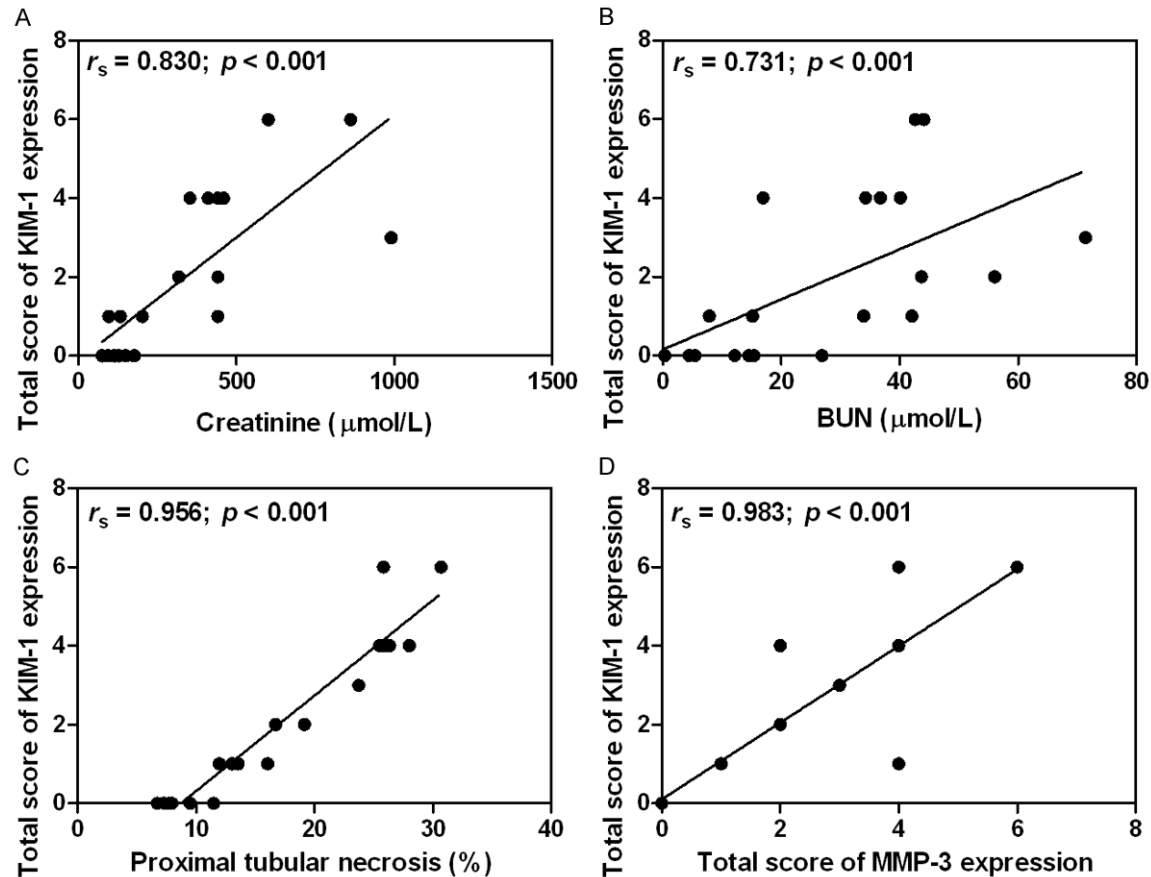
pattern of KIM-1 is demonstrated in **Figure 1**. KIM-1 expression was not detected in normal kidney tissues (**Figure 1A** and **1B**), and rarely demonstrated in kidney tissues from non-AKI group (**Figure 1C** and **1D**). KIM-1 positive tubules were frequently presented in tubular cells with disrupted cellular membrane and in areas with no visible cellular brush border in the AKI group (**Figure 1E** and **1F**). In addition, KIM-1 expression was absent in the glomeruli and inflammatory cells, including lymphocytes, monocytes, and plasma cells in all groups (**Figure 1**). Immunohistochemical staining for MMP-3 revealed a positive staining with brown granular pattern in the cytoplasm of the proximal tubular cells presenting with necrosis. MMP-3 positive staining was frequently expressed in the AKI group (**Figure 2E** and **2F**). Similar to the KIM-1 expression, MMP-3 positive staining was not detected in the glomeruli and inflammatory cells in malaria and normal kidney tissues (**Figure 2**).

For semi-quantification analysis, the mean score of KIM-1 in kidney tissues was significantly increased in AKI group ( $3.60 \pm 0.52$ ), compared to non-AKI ( $0.30 \pm 1.15$ ), and control groups ( $0.00 \pm 0.00$ ) (all  $P < 0.001$ ). The mean score of MMP-3 in kidney tissues was significantly increased in AKI group ( $3.20 \pm 0.47$ ), compared to non-AKI ( $0.3 \pm 0.48$ ), and control groups ( $0.00 \pm 0.00$ ) (all  $P < 0.001$ ). The mean intensity of KIM-1 and MMP-3 expression was highest in AKI group, compared to non-AKI, and control groups (all  $P < 0.001$ ). Importantly, this study demonstrated that an increased expression of KIM-1 is paralleled with an increased expression of MMP-3. The total scores of KIM-1 and MMP-3 expression were significantly increased in AKI group, compared to non-AKI, and control groups (**Figure 3**).

### Correlations between KIM-1 and important parameters

In the kidney tissues of patients with *P. falciparum* infection ( $n = 20$ ), there were significant positive correlations between total score of KIM-1 expression and serum Cr level ( $r_s = 0.830$ ;  $P < 0.001$ ) and serum BUN ( $r_s = 0.731$ ;  $P < 0.001$ ) (**Figure 4A** and **4B**). In addition, strong positive correlation was found between total score of KIM-1 expression and the proximal tubular necrosis ( $r_s = 0.956$ ;  $P < 0.001$ ) and total score of MMP-3 expression ( $r_s = 0.983$ ;  $P$





**Figure 4.** Direct positive correlations between KIM-1 expression and serum Cr (A), serum BUN (B), proximal tubular necrosis (C) and MMP-3 expression (D).

< 0.001) (Figure 4C and 4D). However, no correlation was observed between total score of KIM-1 expression and age ( $r_s = 0.299$ ;  $P = 0.201$ ), and parasite count ( $r_s = 0.183$ ;  $P = 0.440$ ).

### Discussion

Kidney injury molecule-1 (KIM-1) is a type 1 transmembrane glycoprotein with extracellular domain that contains an immunoglobulin like domain and mucin domain [10]. The cytoplasmic domain of KIM-1 is short containing a phosphorylated site responsible for molecular signaling. The ectodomain is cleaved by a metalloproteinase and can be detected in the urine [10]. The present study confirms that KIM-1 expression was localized in the proximal tubular cells with injury in the kidney tissues with AKI, whereas, KIM-1 expression was absent in the glomeruli and inflammatory cells in all groups. This finding is consistent with the previous studies which reported the up regula-

tion of KIM-1 expression in proximal tubules after ischemia and nephrotoxicant injury in rat kidneys [12, 18] and in acute tubular necrosis (ATN) in human kidney [11]. KIM-1 negative staining has previously been shown in glomeruli [11, 19, 20], small blood vessels [21] and inflammatory cells [21]. In addition, humans KIM-1 expression has been reported in other clinical settings such as urate nephropathy [22] and renal cell carcinoma [23]. In ischemic injury, increased expression of KIM-1 has been demonstrated predominantly in the S3 portion of the proximal tubules in response to a variety of injuries or as a result of dedifferentiation of the tubular epithelial cells [18, 24]. In acute renal damage, KIM-1 gene and protein products are up-regulated 3 hr after experimental renal ischemia-reperfusion injury [14]. In rats exposed to 100 mg/kg of gentamycin as nephrotoxicant, KIM-1 positive staining was detected at apical surface of individual cells of the S1/S2 segment at 24 hr after final injection,

whereas at 72 hr after the final injection, the apical positive staining become diffuse within the entire cytoplasm of tubular epithelial cells of the S1/S2 segments at site of necrosis and apoptosis, suggesting that KIM-1 expression might be associated with time- and dose-dependent [25]. In malarial AKI, the apoptosis was demonstrated in the tubular cells and correlated with ATN [7]. It was suggested that KIM-1 expression can transform tubular epithelial cells to active phagocytes, which are capable of internalizing apoptotic and necrotic renal epithelial cells after injury [26]. Further investigations are necessary to study the functional roles of KIM-1 in malarial AKI. The present study revealed a significant positive correlation between the KIM-1 expression and the percentage of proximal tubular necrosis. It can be noted that KIM-1 tissue staining is a sensitive and specific marker of early proximal tubular injury and correlates with the degree of renal dysfunction [21]. This finding confirms that KIM-1 expression is localized on the injured proximal tubular epithelial cells, indicating that the increased expression of KIM-1 might be involved in the pathogenesis of tubular cell damage and repair in AKI with severe *P. falciparum* malaria. Our data demonstrated that direct positive correlation was found between tissue KIM-1 expression and important parameters of AKI, including serum Cr and BUN. This assumption was similar to a study on kidney transplant biopsies [21, 27]. KIM-1 tissue staining has been reported as a sensitive and specific marker of early proximal tubular injury and correlates with the degree of renal dysfunction in kidney transplants with interstitial fibrosis and tubular atrophy [27]. An experiment on animal model indicated that KIM-1 is a better AKI indicator than serum Cr to predict proximal tubule injury [12]. Therefore, this observation support that KIM-1 may serve as a biomarker for malarial AKI.

In addition, this study found that the MMP-3 expression was significantly demonstrated in the kidney tissues of AKI group and was positively correlated with tissue KIM-1 expression. These finding are consistent with a previous study demonstrating the expression of MMP-3 and KIM-1 in the S3 segments of proximal tubules in the kidney after ischemia-reperfusion injury [15]. Several reports demonstrated that shedding of KIM-1 ectodomain is cleaved

by metalloproteinase-dependent process, namely MMP-3 [10, 15]. However, the mechanism of MMP-3 activation for KIM-1 shedding in AKI is still unclear. It has been demonstrated that MMP-3 might be directly activated by mitogen-activated protein kinase (MAPK) [28] or by reactive oxygen species (ROS) [15], resulting in KIM-1 ectodomain shedding. According to physiologic stimuli mediated KIM-1 shedding, pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) has been reported as stimulator for activating MMP-3 expression and mediating apical KIM-1 shedding. During malaria infection, the increasing serum and plasma TNF- $\alpha$  levels were reported in patients with severe malaria [29, 30]. It is possible that the TNF- $\alpha$  might be involved in tubular injury and could activate MMP-3 expression and mediate KIM-1 shedding in malaria AKI. It is known that the ectodomain of KIM-1 is shed from the renal tubular cells and this soluble form of KIM-1 has been shown to be useful biomarker for early detection of the tubular injury and predict adverse clinical outcome in patient with AKI [11, 13, 14, 16]. The higher level of KIM-1 was found in urine samples from patients with ischemic ATN compared to the patients with other forms of acute and chronic renal failure [11]. In malaria patients, neutrophil gelatinase-associated lipocalin (NGAL) and KIM-1 play a good diagnostic performance for AKI, whereas urine NGAL had an excellent predictive performance [16]. Our finding suggest that KIM-1 may serve as a novel biomarker for proximal tubule injury but further studies are needed to validate the utility of this marker in patients with malarial AKI.

## Conclusions

This present study demonstrates that upregulation of KIM-1 may serve as a specific marker for proximal tubular injury in malarial AKI. Moreover, MMP-3 was markedly expressed on proximal tubular injury and closely correlated with KIM-1 expression in malarial AKI, indicating the role of KIM-1 shedding. Further studies are necessary to investigate the mechanism and regulation for KIM-1 shedding in molecular level or experimental model of malarial AKI.

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## Disclosure of conflict of interest

None.

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