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PAPER

Elevated expressions of myeloid-related proteins-8 and -14 are danger biomarkers for lupus nephritis

P Tantivitayakul[1,2], T Benjachat[1,3], P Somparn[1,4], A Leelahavanichkul[1,5], V Kittikovit[6], N Hirankarn[1,5], T Pisitkun[1,4] and Y Avihingsanon[1,7]

[1]Center of Excellence in Immunology and Immune-mediated Diseases, Chulalongkorn University, Bangkok, Thailand; [2]Department of Oral Microbiology, Mahidol University, Bangkok, Thailand; [3]Biomedical Science, Interdisciplinary Program, Chulalongkorn University, Bangkok, Thailand; [4]Research Affairs, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; [5]Department of Microbiology, Chulalongkorn University, Bangkok, Thailand; [6]Department of Pathology, Chulalongkorn University, Bangkok, Thailand; and [7]Department of Medicine, Chulalongkorn University, Bangkok, Thailand

Myeloid-related proteins, MRP-8 and -14, which have been identified as molecules that mediate the danger signaling in innate immune response, are also known as the DAMPs (damage associated molecular pattern molecules). The proteins were found in infiltrating macrophages and neutrophils at inflammatory sites. Their expression was correlated with severe forms of glomerulonephritis. Therefore, this study examined whether or not MRP-8 and -14 can be used as biomarkers for identifying severely active lupus nephritis (LN). Total blood leukocyte samples and renal biopsy tissues from a prospective cohort of LN patients were used to determine mRNA and protein expression levels of MRP-8 and -14. The mRNA levels of MRP-8 and -14 in total blood leukocytes were significantly higher in active LN patients than quiescent LN patients and healthy controls. Moreover, the mRNA levels of MRP-8 and -14 in the total blood leukocytes and kidney tissues were significantly correlated with therapeutic response and the mRNA expression levels in the kidney were associated with an early loss of the kidney function. MRP-8 and -14 can be used as non-invasive prognostic biomarkers in patients with LN. *Lupus* (2015) **0,** 1–8.

Key words: Myeloid-related protein; S100; biomarker; innate immunity; lupus nephritis

Introduction

Lupus nephritis (LN) is an autoimmune-mediated type of glomerulonephritis. Its mechanism in inducing renal injury via the immune-complex is well described.[1] Macrophage infiltrations in the renal tissues are commonly observed in LN but its role in the pathogenesis of LN is unclear. These infiltrating cells may be involved in the different cellular processes of the innate and adaptive immune responses.

Little is known about the innate immune response in LN. Myeloid-related proteins (MRPs) such as MRP-8 and -14 have been shown to play an important role in the development of autoimmune nephritis in mice. Both of these proteins are ligands of the toll-like receptor 4 (TLR4) on the monocytes which participate in the development of autoreactive CD8+ T cells.[2] These proteins were identified as "danger signal" or also known as the DAMPs (damage associated molecular pattern molecules). Other names for MRP 8 and MRP-14 are S100A8 and S100A9, respectively. These proteins are found in the cytoplasm of neutrophils and macrophages.[3] Upon activation, these cells will secrete both of these proteins.[4,5] The complex MRP-8/14 protein can bind to the activated endothelium and this adhesion will assist the phagocytes to migrate anywhere in the body.[6,7] This heterodimer can also induce the up regulation of CD11b/CD18 (MAC-1) receptor on neutrophils resulting in an enhanced adhesion of these cells to the endothelium.[8]

The MRP-8 and -14 expressing phagocytes were associated with many autoimmune and cancer diseases.[9–16] The protein levels in systemic lupus erythematous (SLE) patients were positively correlated

Correspondence to: Yingyos Avihingsanon, Renal Division, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Rama4, Bangkok, 10330, Thailand.
Email: Yingyos.a@gmail.com
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with SLE disease activity.[17,18] Both proteins were found in the renal tissues of various types of glomerulonephritis,[19] especially those with the severe form. Biomarkers for LN remain an unmet need for individualized therapy.[20] Since this marker is non-specific for lupus disease, but it could be used as a therapeutic guidance. Thus the authors examined whether the expressions of MRP-8 and -14 in total blood leukocytes and kidney tissues can be used as diagnostic/prognostic markers for predicting the progress of LN.

Materials and methods

Patients

All SLE patients were enrolled into the study by using the 1997 SLE criteria established by the American College of Rheumatology (ACR). The patients were diagnosed by kidney biopsy proved to be ISN/RPS class III/IV LN. All of the patients were treated with the standard regimen and were continuously monitored. Renal tissues and total blood leukocytes were used to quantitate the amount of MRP-8 and -14 mRNA.

This study was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University. Detailed information of the study was provided to the patients prior to obtaining their written informed consent. This study was registered in the Clinical Trials registry (Clinicaltrials.gov ID#NCT01015456).

This study was divided into two parts: cross-sectional and longitudinal. For the cross-sectional study, the total blood leukocytes were collected from 70 subjects. There were 54 LN patients; 33 had active LN whereas 21 had inactive LN. Inactive LN was defined as having quiescent renal involvement for more than 3 months. 16 healthy volunteers were used as the controls. As for the longitudinal study, there were 26 LN patients. These patients were followed for more than 6 months. Blood samples and kidney biopsies were collected before the induction of treatment. Blood was drawn again six months later. The induction of the standard treatment was given for 6 months and was administered either intravenously with cyclophosphamide of 0.75–1 g/m^2 of body surface area per month or orally with mycophenolate mofetil 1500–2500 mg per day.

Classification of LN activity and treatment response

The Systemic Lupus Erythematous Disease Activity Index (SLEDAI) 2000,[21] or the renal SLEDAI (rSLEDAI), was used to calculate the activity of LN. The sum of the four parameters was used to classify LN: hematuria (>5 red blood cells/high-power field), pyuria (>5 white blood cells/high power field), urinary cast (heme-granular or red blood cell cast), and proteinuria (>1 gram/24 hours). Each parameter was scored based on a 4-point scale.

The treatment responses were classified as responder or non-responder by the following clinical criteria: (1) stable or improved renal function, (2) 50% reduction in hematuria to less than 10 RBC per high-power field, and (3) significant reduction in proteinuria for at least 3 months (50% reduction of urine protein to less than 3 g/day if baseline was in the nephrotic range).

RNA extraction and cDNA synthesis

RNA was extracted from renal biopsy specimens and total blood leukocytes. Total blood leukocytes were isolated by centrifugation method after the red blood cells were selectively lysed. Total RNA was purified by RNeasy purification kit (Qiagen, Valencia, CA, USA). The first strand of cDNA was synthesized from 250 ng of total RNA by using MultiScribe^{TM} Reverse Transcriptase enzyme (Applied Biosystems, Carlsbad, CA, USA). The reaction was performed at 45°C for 45 min.

Quantification of mRNA levels for MRP-8 and -14

mRNA levels for MRP-8 and -14 were measured by real-time quantitative polymerase chain reaction. The sequences of the primers were as follows: MRP 8 sense 5′-GAAGAAATTGCTAG AGACCGAGTG-3′, MRP 8 antisense 5′-CGC CCATCTTTATCACCAGAA-3′, MRP-14 sense 5′-AATTCAAAGAGCTGGTGCGAA-3′ and MRP14 antisense 5′-ATGATGAACTCCTCGA AGCTCAG-3′. Each PCR was performed in a 20μL reaction volume composed of 2μL cDNA template, 10μL of SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, California, USA), 0.5 mM forward primer, and 0.5 mM reverse primer. The amplification reaction was performed by using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, California, USA). The levels of target gene expressions, MRP-8 and MRP-9, were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$).[22] 18 s ribosomal RNA was used as the endogenous reference. The dissociation curve analysis confirmed the amplification of one specific product per primer pair.

Immunohistochemistry

Immunohistochemical study was performed on the renal tissue samples from the LN patients. The renal tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Deparaffinized sections and antigen retrieval were done by proteinase K treatment. The sections were incubated in 3% hydrogen peroxide (v/v) to destroy the endogenous peroxidase activity. Nonspecific protein binding was blocked with 1% bovine serum albumin in PBS. Then the renal tissues were incubated with 1:10 diluted monoclonal antibody against MRP8/14 heterodimer (monoclone: 27E10, Abcam Inc., MA, USA) for overnight. Next, a secondary antibody conjugated with peroxidase enzyme from Envision reagent kit (Dako, Carpinteria, CA, USA) was used. The color product of the peroxidase was developed by the 3,5-diaminobenzidine substrate. The nuclei were counterstained with hematoxylin.

Statistical analysis

Statistical analysis was performed by using SPSS software (version 16, SPSS Inc, Chicago, IL). For continuous variables, data were expressed as mean $\pm$ SE. The differences between groups were calculated by Mann–Whitney tests. Correlation coefficients were calculated by Spearman correlation test. The receiver operator characteristic curve analysis of the mRNA levels was used to determine the cutoff levels that maximized the combined sensitivity and specificity of the loss of the renal function within 12 months. The area under the curve was calculated, and the sensitivity and specificity at the selected cutoffs were determined. A p-value of <0.05 was considered statistically significant. Box plot graphs were done by using GraphPad Prism (version 4.03).

Results

Increased mRNA expressions of MRP-8 and -14 in the blood leucocytes during active LN

Total blood leukocytes samples from active LN ($n=33$), inactive LN ($n=21$), and healthy ($n=16$) were studied. The MRP-8 and -14 mRNA levels were measured by quantitative real-time PCR. Both mRNA levels in active LN patients were significantly higher than inactive LN patients and healthy controls (Figure 1). The mRNA levels of MRP-8 and -14 in the blood leukocytes were positively correlated with the disease activity (i.e. urinary protein excretion ($R^2=0.33$, $p=0.02$) and renal SLEDAI score ($R^2=0.33$; $p=0.05$)). Similarly, MRP-14 mRNA levels of the blood leucocytes were positively correlated with the urinary protein excretion ($R^2=0.45$, $p=0.02$) and renal SLEDAI activity ($R^2=0.31$; $p=0.01$).

MRP-8 and -14 mRNA levels in the blood leucocytes were associated with the therapeutic responses to the immunosuppressive drugs

In the longitudinal study, all active LN patients had biopsy-proven ISN/RPS class III/IV LN. Six months of standard immunosuppressive treatment were administered to the patients. The characters of patients who responded to the treatment are shown in Table 1. MRP-8 and -14 mRNA levels were measured at baseline and at 6 months post-treatment. The treatment response was evaluated 6 months post-treatment and according to the criteria described above.

In the treatment responders, the mRNA levels significantly dropped after 6 months of treatment (Figures 2(b) and (d)). On the other hand, there

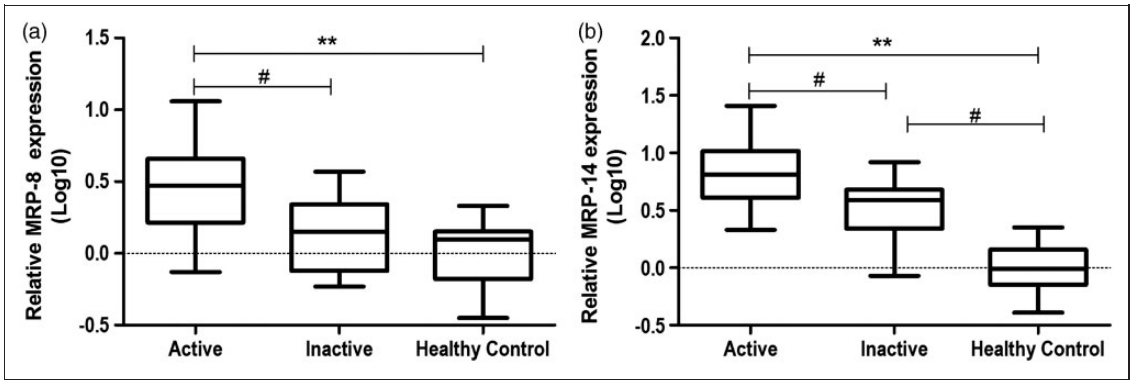

Figure 1 mRNA levels in the blood leucocytes. MRP-8 (a) and MRP-14 (b) mRNA levels among active ($n=33$), inactive LN patients ($n=21$), and healthy controls ($n=16$). The p-value was derived from one-way ANOVA** ($p<0.01$) and t-test $^{\#}$ ($p<0.01$).

Table 1 Characteristics of the patient population from the cross-sectional and longitudinal studies

	Cross-sectional study		*Longitudinal study*[a]	
	Active LN[a]	*Inactive*	*Responder*	*Non-responder*
Age, years				
Mean ± SE	35.4 ± 8.5	31.95 ± 7.37	30.56 ± 2.22	28.53 ± 1.85
Number	33	21	13	13
Prednisone				
Median dosage, mg/day	25	2.5	30	15
Dosage range, mg/day	5–60	2.5–10	15–60	5–45
Renal parameters				
Serum creatinine, mg/dL	1.0 ± 0.45	0.9 ± 0.26	1.1 ± 0.12	1.0 ± 0.45
Proteinuria, g/day	3.8 ± 2.44	0.4 ± 0.19	3.7 ± 0.50	3.5 ± 0.58
Urine erythrocyte count, (HPF)[b]	26 ± 7.9	3 ± 0.57	32 ± 14.46	38 ± 15.73
Renal pathology indices				
Activity index	10.0 ± 0.9	NA	7.8 ± 1.1	8.4 ± 1.4
Chronicity index	3.1 ± 0.5	NA	2.1 ± 0.5	3.1 ± 0.7

[a]Patients were treated with intravenous cyclophosphamide 0.75–1 g/m^2 of body surface area per month for 6 months or orally with mycophenolate mofetil 1500–2500 mg per day for 6 months.
[b]High-power field.

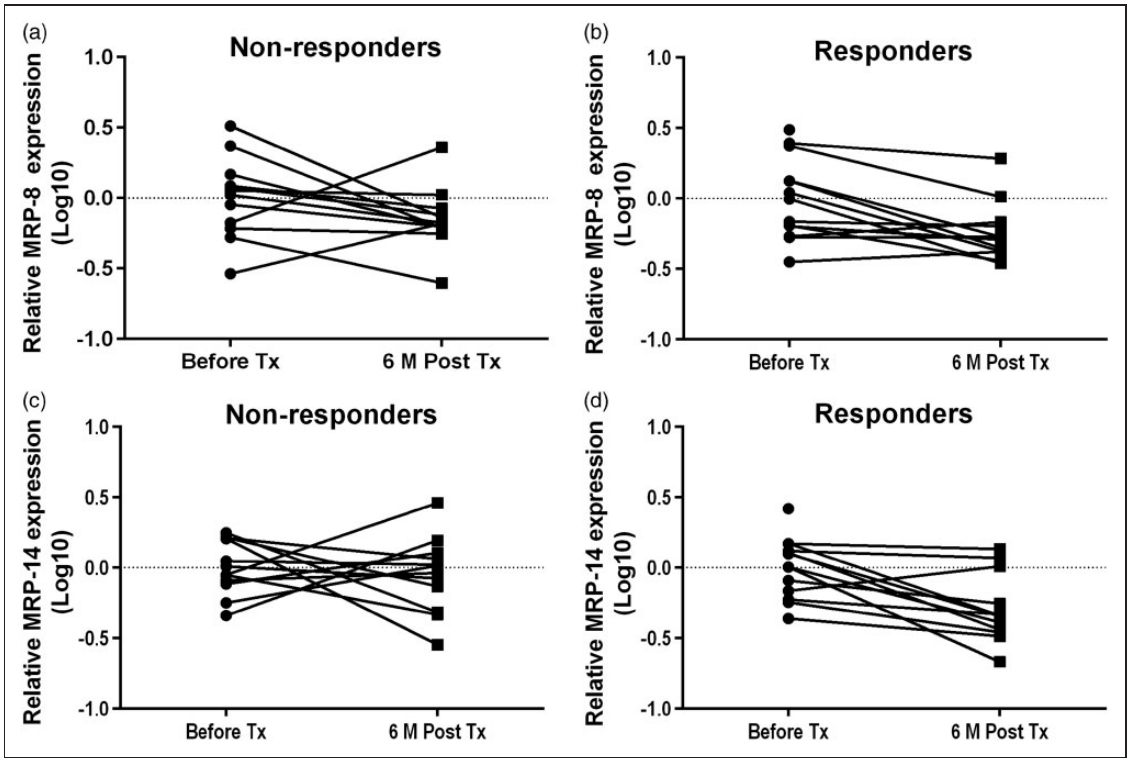

Figure 2 mRNA levels of MRP-8 and -14 in the leucocytes from non- responders (NR) and responders (R). Levels at baseline were compared to the levels after 6 months of treatment. The mRNA levels from the responders were significantly lower after treatment (*$p < 0.05$).

were no changes of the mRNA levels after 6 months of treatment in the non-responders (Figures 2(a) and (c)). The mRNA levels were significantly lower in the treatment responders at 6 months post-treatment compared to the non-responders (MRP-8: -0.33 ± 0.11 vs. -0.15 ± 0.06, and MRP-14:-0.38 ± 0.11 vs. -0.05 ± 0.08, respectively; p-value < 0.05).

Intra-renal mRNA levels of MRP-8 and -14 may indicate the prognosis of the disease

MRP-8 mRNA levels were positively correlated with MRP-14 mRNA levels in the total blood leukocytes as well as in the kidney tissues ($R^2 = 0.58$, $p < 0.01$ and $R^2 = 0.86$, $p < 0.01$, respectively) (see Supplemental figure online)

Next, the authors determined whether the mRNA levels from the renal tissues of LN patients from the longitudinal cohort can predict the therapeutic responses to the standard treatment or not. It should be noted that all cases were biopsy-proven class III/IV LN, and had comparable activity and chronicity indices of the renal histology (Table 1). The mRNA levels in the kidney tissues of the non-responder group were significantly higher than the responder group (Figures 3(a) and (b)) (MRP-8: 0.46 ± 0.14 vs. 0.00 ± 0.09 and MRP-14: 0.41 ± 0.14 vs. 0.00 ± 0.10, respectively; p-value < 0.05).

The authors then determined whether the mRNA levels can predict the loss of renal function within one year. The loss of renal function was defined as having a twofold increment of the serum creatinine from baseline or eGFR less than $15\,mL/min/1.73m^2$. The receiver–operator characteristic (ROC) curves were used to ascertain the best cutoff level that had the maximal sensitivity and specificity to predict the loss of renal function. The cutoff levels of MRP-8 or -14 at 0.5 log had a sensitivity of 65% and a specificity of 88% in predicting when the serum creatinine levels will increase by twofold. As a result of this, the authors then determined the survival analysis when the levels of MRP-8 and -14 were high and low. Patients with high MRP-8 or-14 levels had a significant increased risk of having a doubling of the serum creatinine levels ($p = 0.03$ and 0.02 by log-rank test, respectively) (Figure 4).

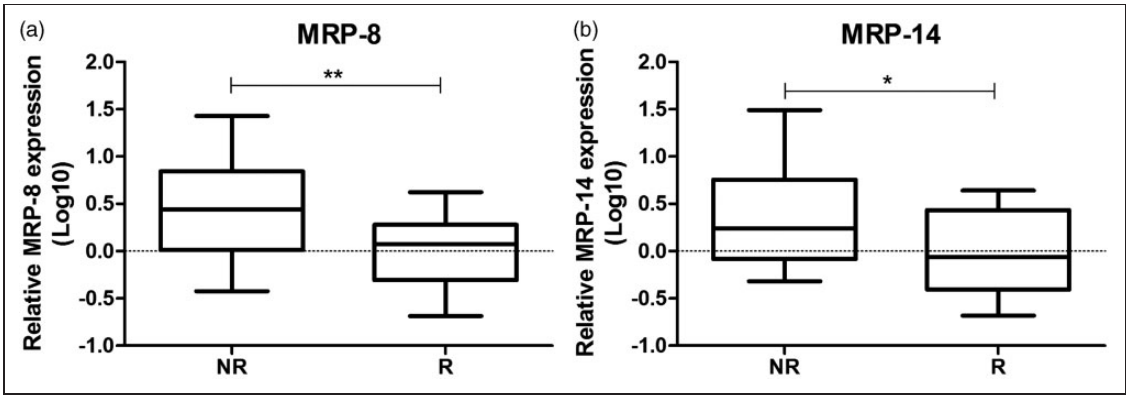

Figure 3 Comparison of intra-renal expressions of MRP-8 and -14 mRNA. MRP-8 (a) and -14 (b) mRNA levels in the kidney tissues of the non-responders (NR) and the responders (R), p-value < 0.05.

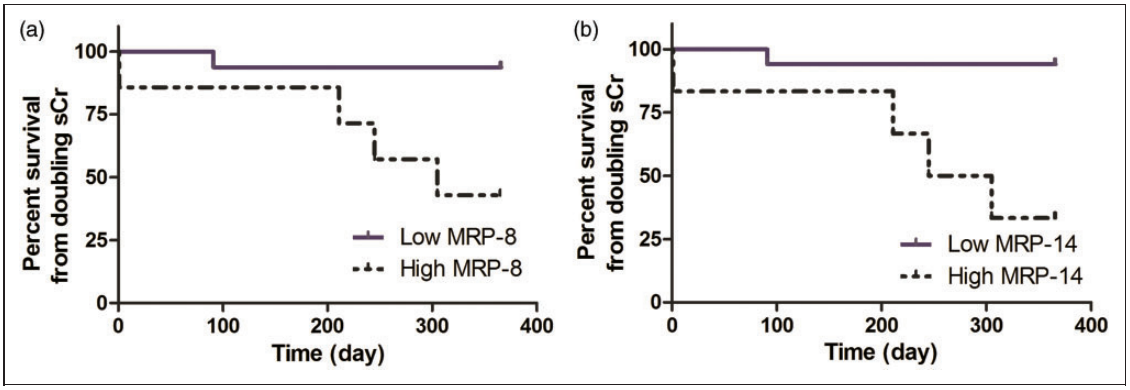

Figure 4 The relationship between the intra-renal MRP-8 and -14 mRNA levels and the loss of renal function within 12 months. Kaplan–Meier estimates of the time to 2-fold increase of the serum creatinine in all patients with class III/IV LN. The best cutoff mRNA level for MRP-8 (a) and -14 (b) identified by the ROC analysis, $p = 0.008$ and 0.002 by log-rank test, respectively.

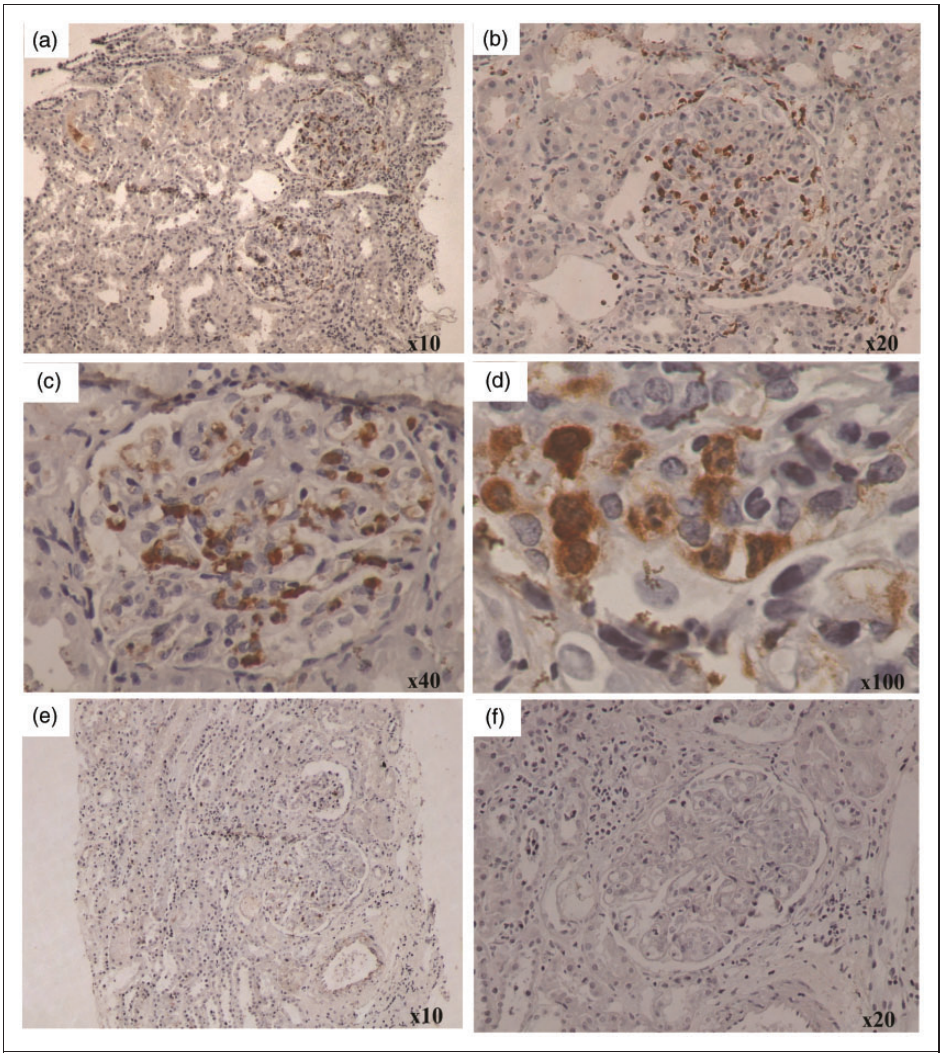

Figure 5 Immunohistochemical staining of the renal tissues from the patients with lupus nephritis (LN). MRP-8/14 positive cells were seen on the glomerular infiltrating cells and peritubular capillaries of the kidney tissues from the non-responders (a–d) but not in the responders (e), and negative control (f). The arrow indicates the cells that have stained positively for MRP-8/14 complex. (×number) represent the magnification power.

MRP-8/14 heterodimer proteins were detected in infiltrating cells of the glomeruli and peritubular capillaries

The localization of the MRP8/14 heterodimer proteins in the renal tissues of LN patients were investigated via immunohistochemistry. Staining with specific antibodies to the heterodimer showed the presence of the MRP-8/14 heterodimer on the infiltrating cells of the glomeruli and peritubular capillaries (Figure 5). This finding corroborated the previous observation that MRP 8/14 expression was detected on the macrophages/neutrophils of lupus kidneys.[19]

Discussion

This study showed the correlation of the DAMPs signatures, MRP-8 and -14, with LN. Their mRNA levels in the total blood leukocyte cells can determine the activity of lupus disease as well as response to treatment. As MRP is a marker of non-specific injury, this marker should only be used in the proven case of SLE with nephritis. Elevated levels of MRP-8 and -14 mRNA in the total blood leukocytes and the renal tissues indicated that there was treatment failure. Furthermore, the presence of these proteins in the

infiltrating inflammatory cells of the glomerulus indicated active LN. Therefore MRP-8 and -14 are good diagnostic and prognostic markers for detecting severely active LN.

MRP-8 and -14 are calcium-binding receptors on leukocytes and macrophages, and represent the innate immune responses. Both are expressed in a heterodimeric form on activated macrophages and associated with Toll-like receptor-4 (TLR-4) signaling. In addition, MRP-8/14 is important in activating the adaptive immune response through CD8 auto-reactive lymphocytes.[2] Since both proteins are important innate signals for the development of autoimmune nephritis in mice, they are possible pathogenic factors for severity of patients with LN.

LN with ISN/RPS class III/IV renal histology will have a rapid decline of the kidney function. Aside from the kidney pathology, there are no other prognostic factors that can predict the progression of LN. Even the most severe form of LN class IV must rely on the presence of the crescentic glomeruli to determine whether the prognosis will be bad or not but this is also dependent upon the percentages of glomeruli involved and can vary from case to case. However, one of the limitations of pathology is dependent upon the site where the kidney biopsy is performed. Thus the results from pathology are not always consistent and sometimes may be due to chance because the sample is randomly and inconsistently pulled out of the kidney. As a result of this, other noninvasive procedures are preferred such as the detection of biomarkers in the blood or urine. A more accurate prognostic test is needed for clinical use. Potential biomarkers for LN include urine mRNAs of cytokines and growth factors. Urine proteins such as pro-inflammatory cytokines and glycoprotein were reported to determine the activity of the disease. Unfortunately, not many biomarkers were proposed to be used as prognostic biomarkers for LN nor could be used to predict the therapeutic responses during treatment. In this study, MRP-8 and -14 mRNA in the blood leucocytes can be used to determine the activity of LN as well as the risk for treatment failure. This technique requires only blood samples which can be acquired easily and repeatedly. Hence, the authors highly recommend that MRP-8 and -14 be developed into a non-invasive biomarker tests for active LN.

A number of studies have used MRP-8/14 biomarker to detect autoimmune diseases or cancer.[23,24] Many have shown that the MRP-8/14 levels were positively correlated with the severity of inflammation as shown in rheumatoid arthritis,[9] glomerulonephritis,[19] as well as with invasion of breast cancer.[13] MRP-8 and -14 can induce the loss of cell junction protein and increase permeability of the endothelial cells. Hence they can cause endothelial damage and result in vasculitis.[25] In this study, MRP-8 and -14 were strongly correlated in both the blood and kidney tissue for LN. Moreover, both mRNAs and proteins were positively correlated with the activity of LN in the kidney. This information supports previous reports of MRP-8/14 complex being responsible for directly inducing the inflammation process.[26,27]

The MRP-8/14 complex protein can amplify the inflammatory response by inducing the extravasation of the neutrophils and macrophages into the inflamed tissues. Mediators of inflammation such as IFN-γ and TNF-α will upregulate the MRP-8/14 protein expression and cause the infiltrating cells to secrete the protein-complex.[28] At the same time, the protein complex of MRP-8/14 will stimulate the secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) by the monocytes and activate the NF-kβ pathway.[29] Because of this, both MRP-8 and -14 can enhance the inflammation in the endothelium and local tissues. These mechanisms may explain why intra-renal MRP-8/14 can predict ongoing active nephritis and poor treatment outcome in this study.

In conclusion, the findings from this study indicate that MRP-8 and -14 are involved in the inflammation process of LN, like other severe form of glomerulonephritis. Their mRNA levels in the blood leucocytes can be used to monitor the activity of the disease as well as predict the treatment outcome for LN. Future studies utilizing this non-invasive monitoring of MRP-8 and -14 are warranted.

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Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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