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The gene expression of NGAL and TLR9 in glomerulus and tubulo-interstitium of patients with lupus nephritis

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ABSTRACT

***Background*: The role of neutrophil gelatinase associated lipocalin (NGAL) and Toll-like receptor 9 (TLR9) in the pathogenesis of lupus nephritis remain elusive. *Methods*: We quantified the glomerular and tubulointerstitial mRNA expression of NGAL and TLR9 in 42 patients with lupus nephritis (LN group) and 10 controls. *Results*: As compared to controls, LN group had higher glomerular expression of TLR9, and higher tubulointerstitial expression of NGAL and TLR9. Tubulointerstitial NGAL expression significantly correlated with proteinuria (r = 0.492; p = 0.003), renal function (r = –0.386; p = 0.022) and histological chronicity index (r = 0.540; p = 0.004). Proteinuria had significant correlation with glomerular (r = 0.554; p = 0.001) and tubulointerstitial (r = 0.379; p = 0.043) TLR9 expression. Furthermore, there was a significant difference in tubulointerstitial expression of NGAL between treatment response groups. *Conclusion*: There is an increase in intra-renal mRNA expression of NGAL and TLR9 in LN. Although tubulointerstitial expression of NGAL does not correlate with systemic disease activity, it correlates with proteinuria, renal function, and therapeutic response. The role of NGAL in the pathogensis in LN, as well as its application as biomarker for lupus nephritis, requires further study.**

Keywords: Lupus Nephritis; Immunology; Cytokine

1. INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by aberrant cytokines milieu. Although almost all organs in the body could be involved, lupus nephritis (LN) remains the leading cause of morbidity and mortality in SLE. The precise pathogenic mechanism of SLE and LN, however, is not completely understood. Cytokine aberration is a cardinal phenomenon of LN. It is important to realize that cytokines may not only be involved in the generation of aberrant immune regulation [1], but also participates in the local inflammatory processes that ultimately lead to tissue destruction. Unfortunately, most studies focused on the cytokine profile of peripheral blood in SLE patients, and the results are often inconsistent [2,3]. Since specific organ or tissue involvement in SLE probably involve local cytokine aberrations that do not appear in the systemic circulation, study of the immunopathogenesis should focus at the specific sites of disease involvement.

Neutrophil gelatinase associated lipocalin (NGAL) is a member of the lipocalin superfamily, which is first found in the specific granules of human neutrophil [4]. NGAL is a promising biomarker for both acute kidney injury (AKI) and chronic kidney disease (CKD) [5]. Urinary NGAL levels correspond to disease activity in lupus nephritis more closely than standard markers [6]. Toll-like receptor 9 (TLR9) is a pattern-associated receptor functioning in innate immunity that recognizes unmethylated CpG sequences in DNA molecules; it may be involved in the recognition of self DNA-antigens and the production of pathogenic auto-antibodies. Previous study showed that a higher expression of TLR9 on peripheral blood B cells from patients with active SLE correlated with SLEDAI [7]. Notably, TLR9 causes the activation of NF-κB [8], which is recruited to the NGAL promoter and is essential for the transcriptional activa-

tion of NGAL promoters [9]. We hypothesize that intra-renal TLR9 and NGAL are related to the development of LN.

2. PATIENTS AND METHODS

2.1 Patient Selection

We studied 42 consecutive SLE patients with active renal disease and required kidney biopsy. All patients fulfilled the American College of Rheumatology diagnostic criteria of SLE [10]. The uninvolved pole of 10 kidneys that were removed for renal cell carcinoma and had no morphological evidence of renal disease were used as control. The study was approved by the Clinical Research Ethical Committee of the Chinese University of Hong Kong; all patients provided informed consent.

2.2. Clinical and Histological Assessment

The disease activity of SLE was assessed clinically by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [11] on the day of kidney biopsy by an independent physician. Baseline serum creatinine, urine protein, complement levels (C3 and C4) and anti-double strand (ds) DNA antibody titre were also measured. Glomerular filtration rate (GFR) was estimated by a standard equation [12].

Kidney biopsy specimen was evaluated according to the International Society of Nephrology (ISN) classification of lupus nephritis [13]. The activity index (AI) and chronicity index (CI) of each biopsy specimen were scored by standard methods [14]. Briefly, AI is the sum of semiquantitative manual scores (0-3 each) of the following parameters: Endocapillary hypercellularity; leucocyte infiltration, subendothelial hyaline deposits; interstitial inflammation, necrosis and cellular crescents. Scores of the last two parameters are counted double, making a total AI of 0 to 24.

2.3. Laser Microdissection

The method of laser microdissection has been described in our previous studies [14,15]. Briefly, cryosections of 10 μm thickness were prepared on a cryostat (Leica Microsystems, Wetzlar, Germany) using disposable microtome blades (Leica Microsystem) in RNase-free conditions, and were mounted on MembraneSlide 0.17 PEN slides (Carl Zeiss PALM Microlaser Technologies, Bernried, Germany). Immediately after taking the slides out of the cryostat, the sections were fixed in 70% ethanol and dehydrated in 100% ethanol. Sections were air-dried at room temperature.

Laser microdissection of the snap-frozen kidney biopsy specimens was performed using the PALM Microlaser System (PALM Microlaser Technologies), which is equipped with a pulsed high-quality laser beam, computer-controlled microscope stage and micromanipulator. Under direct visual control, areas of interest in the histological specimens were selected through the PALM RoboSoftware (PALM Microlaser Technologies) by moving the computer mouse, and microdissected by cutting the contour of the selected areas with the adjusted laser beam. The isolated tissue was then laser-catapulted into a microcentrifuge cap filled with guanidine thiocyanate containing lysis buffer for the subsequent RNA isolation. Approximately 20-30 glomerulus and 20 randomly selected tubulointerstitial area were isolated from each specimen. The tissue lysate of glomerulus and tubulointerstitium were kept at –80˚C until RNA extraction was performed with the RNAqueous®-Micro Kit (Applied Biosystems, USA), following manufacturer's instruction.

2.4. Quantification of Intrarenal Gene Expression

The method of total RNA extraction, reverse transcription and real-time quantitative polymerase chain reaction (RT-QPCR) was described in our previous studies [14,16]. In the present work, we quantified the mRNA expression of NGAL and TLR9 in glomerulus and tubulointerstitium. Taqman primers and probes of each target were purchased from Applied Biosystems (Foster City, California, USA). The RT-QPCR was performed by ABI Prism 7900HT Sequence Detector System (Applied Biosystems) following the manufacturer's instruction. The mRNA expression for each signal was calculated by using the ΔCt procedure according to manufacturer's instruction, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as the housekeeping gene for normalisation among samples. All primers and probes were tested with purified DNA as the template in RT-QPCR to ensure that they do not amplify genomic DNA. All results were analysed by Sequence Detection Software version 1.7 (Applied Biosystems).

Gene expression of each target was calculated by using the difference-in-threshold-cycle (ΔCt) procedure, according to manufacturer's instruction. For GAPDH and each target, the relative efficiency of amplification over various starting template concentrations was determined. Approximately equal efficiencies for other targets with GAPDH amplifications were verified by an absolute value of <0.1 for the slope of log input complementary DNA amount versus ΔCt, which was obtained by subtracting the threshold cycle (Ct) value of GAPDH from that of the target. Therefore, it was possible to detect GAPDH in the same tube with other targets. The relative quantification of using multiplex reaction with a comparative method was determined by the formula $2^{-(\Delta\Delta Ct)}$, where the ΔΔCt was calculated by the subtrac-

tion of ΔCt of the calibrator from ΔCt of the sample.

2.5. Clinical Follow-Up

After the renal biopsy, all patients were followed for at least 6 months. The clinical management was decided by individual nephrologist and not affected by the study. In general, patients were treated with corticosteroid, together with cyclophosphamide or mycophenolate according to published protocols. Therapeutic response to induction immunosuppressant was assessed at 12 weeks after treatment and classified into complete remission, partial remission, and no response as defined previously [17]. Briefly, complete remission was defined as a value for urinary protein excretion that was less than 0.3 g/24 hours, with normal urinary sediment, a normal serum albumin concentration, and values for both serum creatinine and estimated GFR that were 15% or less above the base-line values. Partial remission was defined as a value for urinary protein excretion that was between 0.3 and 2.9 g/24 hours, with a serum albumin concentration of at least 30 g/dL and stable renal function. Treatment failure was defined as a value for urinary protein excretion that remained at or above 3 g/24 hours or a value of 0.3 to 2.9 g/24 hours but with a serum albumin below 30 g/dL, an increase in the serum creatinine concentration ≥ 50 μmol/l, or a value for estimated GFR that was more than 15% above the base-line value, or the discontinuation of treatment due to side effects.

2.6. Statistical Analysis

Statistical analysis will be performed by SPSS for Windows software version 15.0 (SPSS Inc., Chicago, IL). All the results from this series of experiments are quantitative. The results are presented as mean ± SD unless otherwise specified. Since the data on gene expression are highly skewed, they are compared between groups by Kruskall Wallis test or Mann-Whitney U test as appropriate. Correlations between continuous variables are calculated by Spearman's rank correlation coefficient. A p value of less than 0.05 is considered as statistically significantly. All probabilities are two tailed.

3. RESULT

We studied 42 SLE patients. Their baseline demographic and clinical data are summarized in **Table 1**. The histological diagnoses were proliferative nephritis (class III or IV, 9 cases), pure membranous nephritis (class V, 9 cases), class II nephritis (3 cases), and mixed proliferative and membranous nephritis (21 cases). The mean histological Activity and Chronicity Indices were 7.1 ± 4.3 and 2.7 ± 2.2, respectively.

Table 1. Baseline demographic and clinical data.

	Patient group	Control group
No. of patient	40	10
Sex (M:F)	2:40	4:6
Age (years)	40.8 ± 11.4	36.4 ± 13.7
SLEDAI score	7.6 ± 4.5	0
Serum creatinine (μmol/l)	125.4 ± 115.5	82.7 ± 14.0
Proteinuria (g/day)	2.33 ± 1.84	0.01 ± 0.03
Glomerular filtration rate (mL/min/1.73m^2)	71.2 ± 37.2	106.6 ± 22.0
Renal histology		
Interstitial fibrosis (%)	13.8 ± 17.9	1.4 ± 2.344
Glomerosclorosis (%)	10.8 ± 15.5	2.6 ± 5.3

SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

3.1. Gene Expression Level between Groups

The glomerular and tubulointerstitial mRNA expression levels of NGAL and TLR9 are summarized in **Figure 1**. As compared to controls, LN patients had higher glomerular expression of TLR9 ($p < 0.001$) but not NGAL. In contrast, LN patients had higher tubulointerstitial expression of TLR9 ($p = 0.011$) and NGAL ($p < 0.001$) as compared to controls. There was a modest internal correlation between tubulointerstitial mRNA expression levels of NGAL and TLR9 ($r = 0.386$; $p = 0.047$). The glomerular and tubulointerstitial expression of NGAL are also significantly correlated ($r = 0.410$; $p = 0.03$), while those of TLR9 had no significant correlation ($r = 0199$, $p = 0.388$). There were no significant differences in glomerular or tubulointerstitial expression of TLR9 or NGAL between histological groups (details not shown).

3.2. Relation with Clinical and Histological Parameters

We further explore the correlation between gene expression and baseline clinical and histological parameters within patients with lupus nephritis. We found that tubulointerstitial, but not glomerular, NGAL significantly correlated with proteinuria ($r = 0.492$; $p = 0.003$), estimated GFR ($r = –0.386$; $p = 0.022$) and histological CI ($r = 0.54$; $p = 0.004$) (**Figure 2**). In contrast, both glomerular ($r = 0.554$; $p = 0.001$) and tubulointerstitial ($r = 0.379$; $p = 0.043$) TLR9 expression significantly correlated with proteinuria, but not estimated GFR or histological CI (**Figure 3**). The glomerular or tubulointerstitial expressions of TLR9 or NGAL did not correlate with markers of systemic disease activity, including SLEDAI score, serum complement, or anti-ds DNA titre (details not shown).

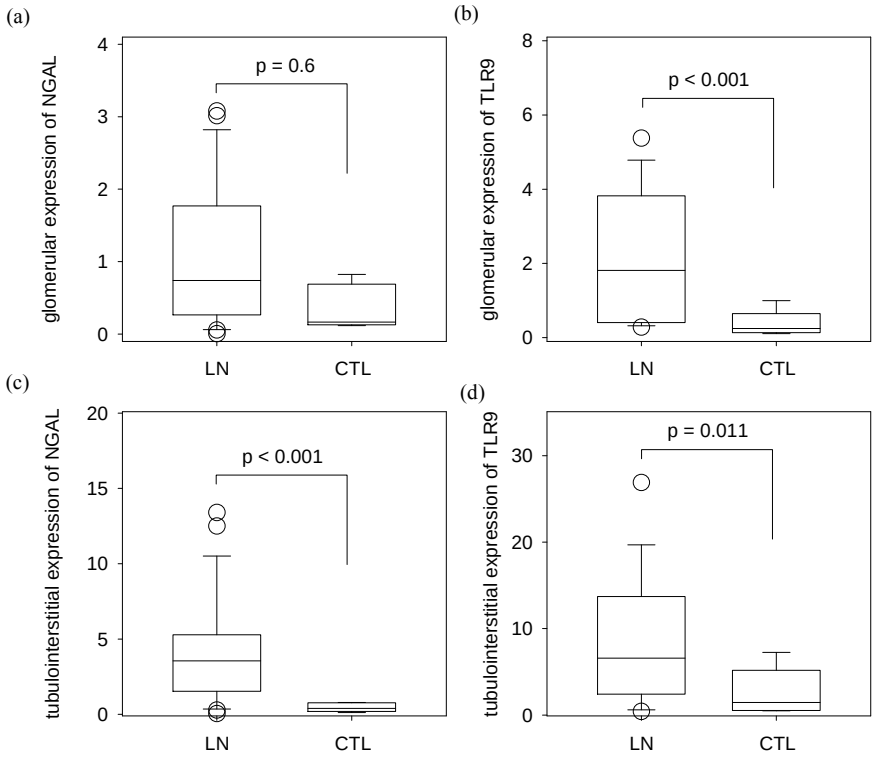

Figure 1. Comparison of glomerular expression of (a) NGAL and (b) TLR9, and tubulointerstitial expression of (c) NGAL and (d) TLR9, between groups. Whisker-box plot, with the boxes indicate median, 25 and 75 percentile; whisker caps indicate 5 and 95 percentile; circles indicate outliers. Data are compared by Mann-Whitney U test. (LN, lupus nephritis group; CTL, control group)

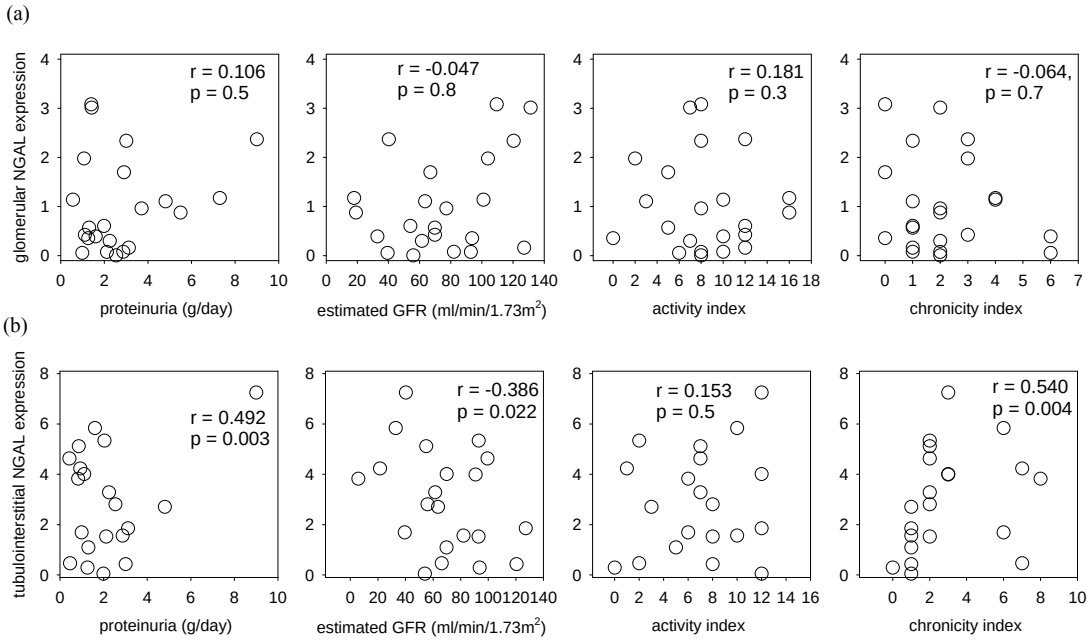

Figure 2. Correlation between (a) glomerular and (b) tubulointerstitial expression of NGAL and the degree of proteinuria, estimated glomerular filtration rate (GFR), histological activity index and chronicity index. Data are compared by Spearman's correlation coefficient. (Some of the circles overlap; each circle on the figure may represent more than one patient.)

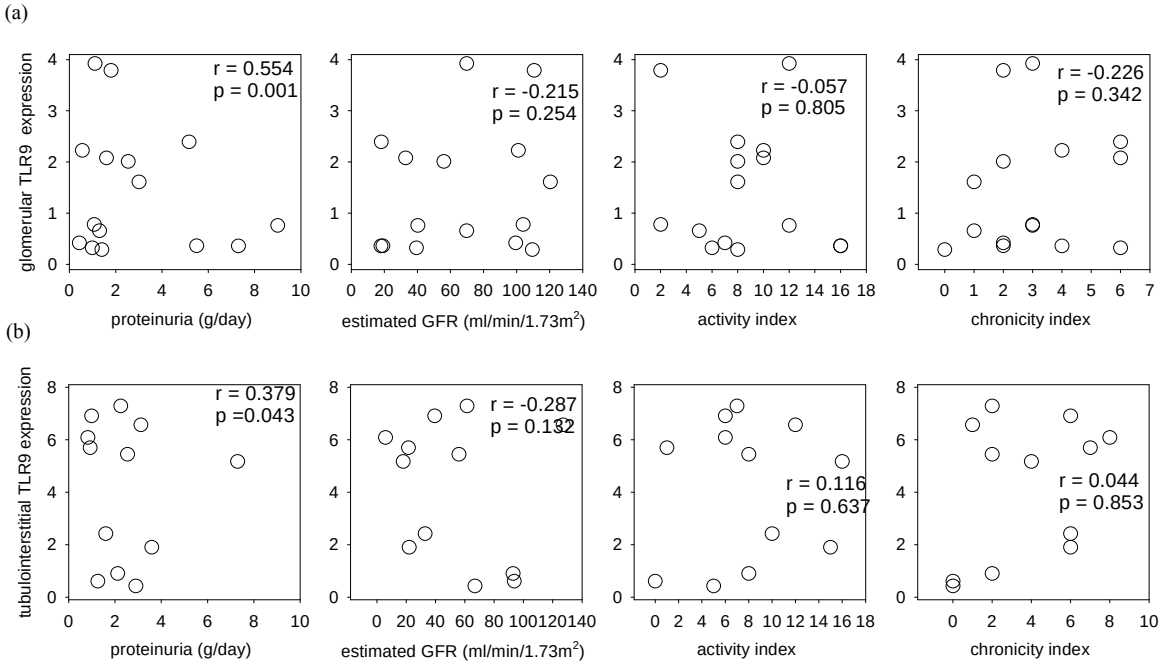

Figure 3. Correlation between (a) glomerular and (b) tubulointerstitial expression of TLR9 and the degree of proteinuria, estimated glomerular filtration rate (GFR), histological activity index and chronicity index. Data are compared by Spearman's correlation coefficient. (Some of the circles overlap; each circle on the figure may represent more than one patient.)

3.3. Relation with Therapeutic Responses

After 6 months follow up, 14 cases had complete remission (CR), 21 cases had partial remission (PR) cases and 7 cases had no response (NR). The expressions of the two target genes are compared between treatment response groups (**Figure 4**). There was a significant difference in tubulointerstitial expression of NGAL between treatment response groups. Post hoc analysis showed that, as compared to CR group, NR group had lower tubulointerstitial expression of NGAL ($p = 0.039$). There was no significant difference in the glomerular expression of NGAL, or glomerular or tubulointerstitial expression of TLR9, because treatment response groups.

4. DISCUSSION

In the present study, we found that NGAL expression only increased in the tubulointerstitium of LN. Our result is similar to that of Brunner *et al.*, who found significantly greater urinary NGAL levels in individuals with active renal disease as compared to others [18]. Our findings also suggest that NGAL expression was abundant in tubulointerstitium but not glomerulus, which is consistent with the general belief. Furthermore, we found that tubulointerstitial NGAL expression significantly correlated with proteinuria, GFR and CI. The result agrees with the report of Bolignano *et al.*, who also showed that urinary NGAL concentrations correlated with renal function and proteinuria [19]. In addition, we found that the tubulointerstitial expression of NGAL is related to treatment response. Our results concur with the report of Hinze *et al.* [20], which also showed that urinary and plasma NGAL are predictors of the course of LN in childhood-onset SLE patients. Taken together, the data suggest that the alteration of tubulointerstitial NGAL in LN is functionally relevant.

The site of renal TLR9 expression has been a matter of debate. In a recent study, Machida *et al.* [21] reported injured glomerular podocytes in active LN expressed TLR9, which disappeared during remission and was not expressed in a normal control kidney. Furthermore, TLR9 expression correlated with the degree of proteinuria and anti-dsDNA antibody titre. On the other hand, Benigni *et al.* [22] showed a robust TLR9 expression in the proximal tubular epithelial cells in an animal lupus model and patients with LN, and TLR9 level correlated with proteinuria and tubular damage. Our result showed that TLR9 expression increased in both glomerulus and tubulointerstitium of LN, which is consistent with the report of Papadimitraki *et al.* [23].

The role of TLR9 in LN is relatively well studied. The up-regulation of TLR9 in the renal parenchyma of LN may be a response to inflammatory cytokines or the nucleosomes present within the lesion [23]. In return, TLR9 induces macrophages and B-cells to produce cytokines and chemokines, thus aggravates inflammation and tissue damage [24-26]. Studies showed that inter

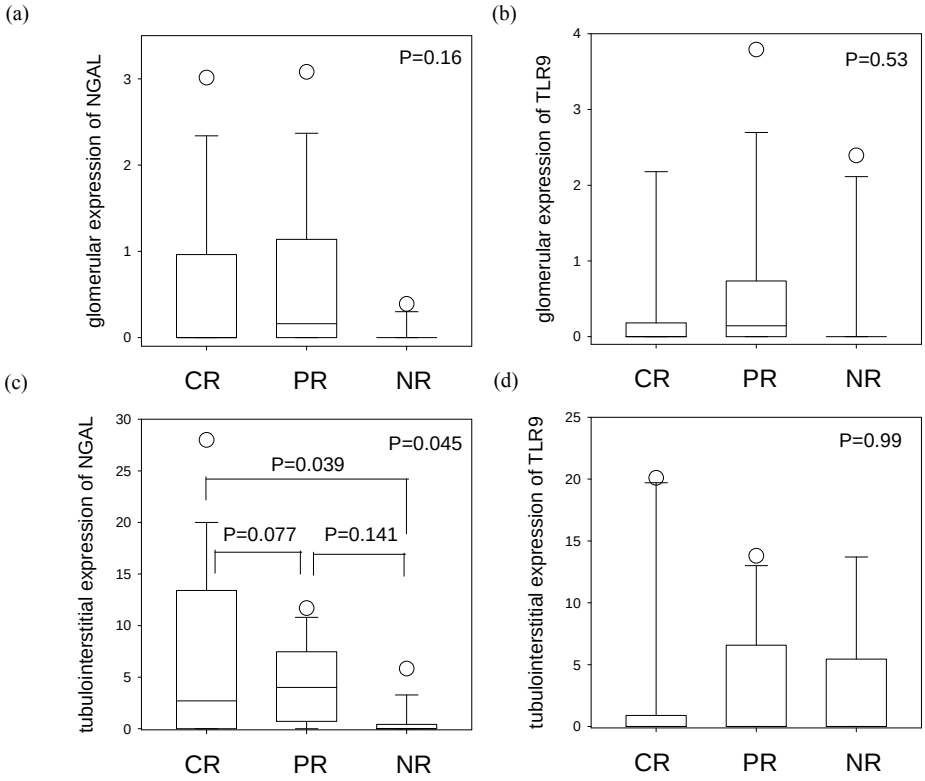

Figure 4. Comparison of glomerular expression of (a) NGAL and (b) TLR9, and tubu lointerstitial expression of (c) NGAL and (d) TLR9, between treatment response groups. Data are compared by Kruskal Wallis test. The box indicates median, 25 and 75 percentile; whisker caps indicate 5 and 95 percentile; outliers are represented as closed circles. (Key for response groups: CR, complete remission; PR, partial remission; NR, no response.)

nalization of SLE immune complexes into subcellular lysosomes containing TLR9 induced a signaling cascade that led to the activation of dendritic cells and production of cytokines and chemokines [27], suggesting that TLR9 may have a role in the onset and progression of LN. In the present study, we found a modest, although statistically significant, correlation between tubulointerstitial expression of NGAL and TLR9. Our result indicates that tubulointerstitial NGAL expression is governed by factors other than TLR9.

Several limitations of the current study need to be addressed. First, the sample size of our study was small, so that subgroup comparison of gene expression between different histological classes of lupus nephritis may not be reliable. Because of the limitated amount of pathological specimen available, we did not quantify intra-renal TLR9 or NGAL at protein level by immunohistochemistry or western blotting. In addition, we could not examine renal tissue of lupus patients without kidney problem as the control group. Furthermore, we do not examine the longitudinal change of intra-renal gene expression or the response to immunosuppressive therapy by serial renal biopsy. Further experiments are necessary to solve these limitations and confirm our findings.

In summary, we found an increase in intra-renal mRNA expression of NGAL and TLR9 in LN. Although tubulointerstitial expression of NGAL does not correlate with systemic disease activity, it correlates with proteinuria, renal function, and therapeutic response. The role of NGAL in the pathogensis in LN, as well as its application as biomarker for lupus nephritis, require further study.

5. ACKNOWLEDGEMENT

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