Measurement of urinary chemokine and growth factor messenger RNAs: A noninvasive monitoring in lupus nephritis

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Noninvasive molecular tests of urine cells have been developed to monitor the activity of kidney diseases. We evaluate whether measurement of urinary messenger RNA (mRNA) levels of chemokine and growth factor genes could distinguish between diffuse proliferative lupus nephritis (class IV LN) and others and whether it is able to predict the response to therapy. Prebiopsy urine samples were collected from 26 LN patients. Urine specimens were serially collected over a period of 6 months from class IV LN patients who were receiving standard immunosuppressive treatments. Urinary interferon-producing protein 10 and its CXC chemokine receptor (CXCR)3, transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) mRNA levels were analyzed by quantitative real-time polymerase chain reactions. Levels of chemokine or growth factor mRNAs in urine could distinguish class IV LN from others, with a sensitivity of 85% and a specificity of 94%. The receiver-operative characteristic curve demonstrated that urine mRNA levels of these genes could identify active class IV LN with an accuracy greater than the current available clinical markers, namely systemic lupus erythematosus (SLE) disease activity index, proteinuria, renal function, or urinalysis. A significant reduction of interferon-producing protein 10 (IP-10), CXCR3, TGF-β, and VEGF mRNA levels from baselines was observed in patients who responded to therapy, whereas the levels tended to increase in those who resisted to treatment. Measurement of urinary chemokine and growth factor mRNAs can precisely distinguish class IV LN from others. Temporal association between these markers and therapeutic response is demonstrated. This noninvasive approach serves as a practical tool in diagnosis and management of LN.

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Clinical manifestations of lupus nephritis (LN) could vary from asymptomatic urinary abnormalities to rapidly progressive renal failure. Severity of LN can fluctuate during the course of the disease and treatment; thus, it requires serial monitoring.[1,2] Although urinalysis or quantification of urinary protein levels is simple and may aid the diagnosis, it cannot distinguish among various LN classes. Assessments of serum auto-antibodies and complement levels also have limited value.[3–5] Knowing the nature or type of glomerular pathology is crucial in predicting the prognosis of the patients. For example, a patient with class IV LN usually turns into an advanced state of chronic kidney disease within a few months, whereas a class II or class V patient may maintain nearly normal renal function.[4] Therefore, kidney biopsy is necessary not only for establishing the diagnosis but also for confirmation of relapse.[3,6] Although essential, repeated renal biopsy is not practical due to its invasiveness and potential unacceptable complications. Therefore, a noninvasive tool to monitor relapse as well as to guide treatment decision is extremely warranted.

Substantial evidence suggests that T-helper 1 (Th-1) type chemokines, in particular γ-interferon-inducible protein (IP-10), contribute to the inflammatory cell infiltration of affected organs in SLE patients.[7,8] IP-10 is expressed and secreted by monocytes and endothelial cells. In a lupus mouse model, this chemokine regulates the Th1-cell migration into kidney and lung via interaction with CXC chemokine receptor (CXCR)3.[8] Neutralizing monoclonal antibodies or small-molecule inhibitors that disrupt CXCR3 function markedly attenuates the inflammatory response, resulting in reduction of kidney damages.[9,10] In human SLE, serum levels of IP-10 protein were high, particularly in patients with active nephritis.[11] The increased urinary levels of monocyte chemoattractant protein-1 and IFN-γ proteins in active LN patients support the pathological

roles of these Th-1 type chemokines.[12,13] Growth and sclerosing factors such as transforming growth factor-β (TGF-β) may also play a pivotal role in the initiation of inflammation and the progression of renal fibrosis.[14–16] TGF-β is a potent inducer of vascular endothelial growth factor (VEGF) expression.[17,18] VEGF can increase vascular permeability and lead to proteinuria. Coexpression of both genes has been demonstrated in renal tissue containing accelerated tissue repair and fibrosis.[19] One study has revealed increased VEGF gene expression in both plasma and renal tissue from LN patients.[20]

Determination of cytotoxic T-lymphocyte messenger RNAs (mRNAs) of urine cells has been shown to be useful in the diagnosis of acute renal allograft rejection.[21] Similarly, urinary mRNAs of some Th-1 cytokines, chemokines, and growth factors correlate with SLE disease activity index (SLEDAI) and clinically defined renal flare. However, this approach cannot precisely distinguish diffuse proliferative LN (class IV LN) from other types.[12,13] Importantly, 15–20% of patients with class IV LN respond poorly to the standard therapy and cannot be predicted by currently available markers.[22–24] Therefore, a new surrogate marker is needed.

Measuring mRNA from urine cells can partly reflect the molecular milieu of the whole kidney.[21,25] Thus, the levels of such gene candidates could be employed to study pathophysiologic mechanisms and disease processes. Fluctuating levels of the urinary mRNA of some genes may be markers of the severity or chronicity of the disease. The present study is aimed to determine whether the mRNA levels of chemokine IP-10 or CXCR3 and growth factor TGF-β or VEGF in urine cells could be applied individually or in combination for routine clinical use.

RESULTS

Patient characteristics

We studied urine samples ($n=52$) at baseline from 26 patients. In all, 10 urine samples from healthy females served as controls. The demographic and clinical data of studied patients are summarized in Table 1. There were 14 patients with class IV, three with class II, three with class III, four with class V, and two with class VI LN. The mean$\pm$standard deviation of baseline pathological activity and chronicity indices for class IV group were 12 ± 6 and 4 ± 4, respectively.

Elevation of urinary chemokine, IP-10, and CXCR3, mRNA levels in class IV LN

The mRNA levels of chemokine, IP-10, and CXCR3 in urinary cells were higher in patients with class IV LN as compared with those in other classes (2.44 ± 0.14 vs 1.06 ± 0.19; $P<0.001$ and 1.81 ± 0.17 vs 0.99 ± 0.16 copies/μg total RNA; $P=0.002$, respectively) (Figure 1a and b). The natural log-transformed chemokine mRNA levels were used as the dependent variable in one-way ANOVA to test the differences between the LN classes. The urinary IP-10 and CXCR3 mRNA levels from patients with class IV LN, measured within 2 weeks before renal biopsy, were significantly higher compared to those of class II, III, V, and VI (Table 2). No significant difference of the housekeeping gene 18s receptor RNA (rRNA) levels was observed among the five

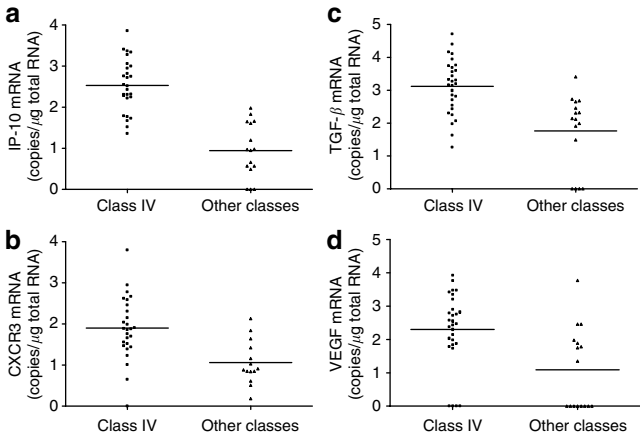

Figure 1 | Levels of mRNA in urine cells. Dots and bars show (log)mRNA levels and means for (**a**) IP-10, (**b**) CXCR3, (**c**) TGF-β, and (**d**) VEGF in urine samples from patients classified as having class IV LN or non-class IV LN. The levels of IP-10 ($P<0.001$), CXCR3 ($P=0.002$), TGF-β ($P<0.001$), and VEGF ($P<0.001$) were significantly higher in patients with class IV than in patients with other classes of LN.

Table 1 | Patient characteristics[a]

	Class IV	Other classes	*P*-value
Number	14	12 (3:3:4:2) (II:III:V:VI)	
Number of urine samples	28	24	
Age (year)	29 ± 1	30 ± 2	0.95
Gender (F/M)	13/1	10	0.90
Serum creatinine (mg/dl)	1.48 ± 0.35	1.44 ± 0.25	0.92
Creatinine clearance (ml/min)	62.9 ± 7.2	48.4 ± 9.4	0.23
Proteinuria (g/day)	2.2 ± 0.5	2.8 ± 0.7	0.43
Urinary erythrocyte count (per high power)	28 ± 9.0	14 ± 6.9	0.22
SLEDAI	6.8 ± 0.9	5.4 ± 1.0	0.32
Steroid dosage (mg/day)	33 ± 6	30 ± 7	0.75

[a]The groups of patients were identified by kidney biopsy based on WHO classification.

Table 2 | Levels of mRNA in urine cells[a,b]

	Class II	Class III	Class IV	Class V	Class VI	*P*-value
IP-10	0.86 ± 0.27	1.55 ± 0.50	2.44 ± 0.14	1.25 ± 0.26	1.1 ± 0.1	< 0.001
CXCR3	1.12 ± 0.26	1.53 ± 0.24	1.81 ± 0.17	0.84 ± 0.20	0.5 ± 0.1	0.005
TGF-β	1.64 ± 0.26	2.0 ± 0.52	3.24 ± 0.13	1.40 ± 0.44	3.03 ± 0.38	< 0.001
VEGF	0.27 ± 0.27	1.91 ± 0.15	2.39 ± 0.20	0.29 ± 0.29	3.03 ± 0.62	< 0.001

[a]Data expressed as mean ± s.e.m. of log copies/μg total RNA.
[b]The groups of patients were identified by kidney biopsy based on WHO classification.
IP-10: interferon-producing protein 10, TGF-β: transforming growth factor-β, VEGF: vascular endothelial growth factor.

groups ($P = 0.56$). The urinary mRNA levels of IP-10 and CXCR3 were not detected in the controls (data not shown).

Elevation of urinary growth factor, TGF-β, and VEGF, mRNA levels in class IV and class VI (diffuse glomerulosclerosis) LN

The mRNA levels of growth factor, TGF-β, and VEGF in urine cells were higher in patients with class IV LN as compared with those in other LN classes (3.24 ± 0.13 vs 1.81 ± 0.26; $P < 0.001$ and 2.39 ± 0.20 vs 0.99 ± 0.29 copies/μg total RNA; $P < 0.001$, respectively) (Figure 1c and d). The urinary TGF-β and VEGF mRNA levels from patients with class IV LN, measured within 2 weeks before renal biopsy, were significantly higher compared with class II, III, and V. However, there was no significant difference between the mRNA levels from class IV and class VI LN (Table 2). Interestingly, the two patients of class VI LN formerly had biopsy-proven class IV LN. Both had a history of multiple nephritis flares and had progressive deterioration of renal function despite aggressive immunosuppressive therapy.

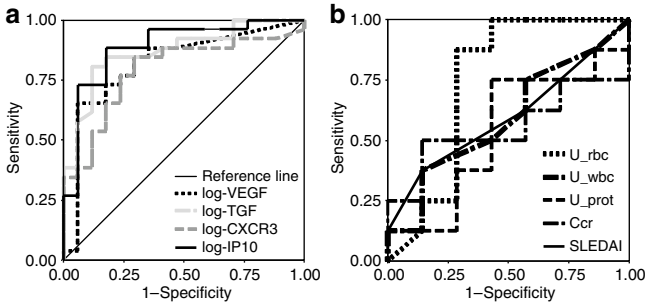

Figure 2 | ROC curves of mRNA levels and other diagnostic markers. The fraction of true-positive results (sensitivity) and that false-positive results (1−specificity) for (**a**) IP-10, CXCR3, TGF-β, and VEGF mRNA levels, and (**b**) other diagnostic markers as markers of class IV LN are shown. The calculated area under the curve was 0.89 for IP-10, 0.79 for CXCR3, 0.87 for TGF-β, 0.82 for VEGF mRNA levels, 0.598 for SLEDAI, 0.554 for creatinine clearance, 0.536 for 24-h urine protein, 0.589 for urine leukocyte count, and 0.741 for urine erythrocyte count. A value of 0.5 (reference line) is no better than expected by chance and a value of 1.0 reflects a perfect indicator.

Urine chemokine and growth factor mRNAs distinguish class IV LN from other LN classes

The receiver-operator characteristic (ROC) curves (Figure 2) depicted the true positive fractions (sensitivity) and false-positive fractions (1−specificity) at various cut points for mRNA levels for IP-10, CXCR3, TGF-β, and VEGF (Figure 2a) and other diagnostic markers: SLEDAI, creatinine clearance, 24-h urine protein excretion, urinary leucocytes, and erythrocytes (Figure 2b). The natural log-transformed cut point (threshold) that maximized the combined sensitivity and specificity for IP10, CXCR3, TGF-β, and VEGF was 2.09, 1.65, 2.50, and 1.82 copies/μg total RNA, respectively. At these thresholds, the sensitivity and specificity were 73 and 94% for IP-10, 65 and 83% for CXCR3, 85 and 83% for TGF-β, and 77 and 76% for VEGF, respectively. The calculated area under the ROC curve for IP-10 was 0.89 (95% confidence interval = 0.78–0.99), for CXCR3 was 0.79 (95% confidence interval = 0.65–0.93), for TGF-β was 0.87 (95% confidence interval = 0.76–0.97), and for VEGF was 0.82 (95% confidence interval = 0.68–0.96). Given the fact that class VI LN can probably be recognized by prebiopsy parameters such as treatment failure and irreversible deterioration of renal function, it might be interesting to assess the usefulness of these markers by leaving out the class VI patients from the analyses. Indeed, removing class VI from the analyses increased diagnostic accuracy of these genes, particularly TGF-β and VEGF. The sensitivity and specificity increased to 85 and 94% for TGF-β and 85 and 84% for VEGF, respectively.

Figure 2b shows the ROC curves for other conventional diagnostic markers with respect to class IV LN. Comparing with the urine mRNA levels, these markers poorly discriminated class IV LN from other classes of LN, as could be demonstrated by the calculated area under the curve at 0.598, 0.554, 0.536, 0.589, and 0.741 for SLEDAI, creatinine clearance, 24-h urine protein excretion, urinary leucocytes, and erythrocytes, respectively.

Pearson correlation analysis revealed that there was a significant correlation between IP-10 and CXCR3 mRNA levels in urine ($R = 0.78$, $P < 0.001$). There was a correlation between TGF-β and VEGF mRNA levels in urine as well (Figure 3a and b) ($R = 0.877$, $P < 0.0001$).

An overlapping of urinary mRNA levels between class IV and other classes (Figure 1) was observed in three patients. Among non-class IV group, two of class VI and one of class III patients showed mRNA levels comparable to those of class IV LN. Using the threshold levels described above, the

patients with class VI LN had high urinary levels of TGF-β and VEGF, but not IP-10 and CXCR3 mRNAs. One patient with class III LN had high urinary levels of all studied genes. Interestingly, this patient had subsequently developed class IV LN despite steroid therapy and required a potent immunosuppressive regimen a few months later.

Serial monitoring of urinary chemokines and growth factor mRNA levels predicts response to therapy

Serial measurements of urine mRNA levels were performed monthly from baseline to the fifth month in 10 responders and four nonresponders of class IV LN according to the criteria described below. The baseline mRNA levels did not significantly differ between these two groups. The IP-10, CXCR3, TGF-β, and VEGF mRNA levels were markedly reduced in the responder group after treatment (Figure 4a) (P-value = 0.01, 0.05, 0.01, and 0.03, respectively). In contrast, the IP-10, CXCR3, TGF-β, and VEGF mRNA levels tended to increase in the nonresponder group (Figure 4b) (P-value = 0.85, 0.66, 0.65, and 0.46, respectively). All patients in the nonresponder group had additional kidney biopsies, which showed worsening of renal pathologies. One of these patients developed crescentic glomerulonephritis with diffuse proliferative nephritis.

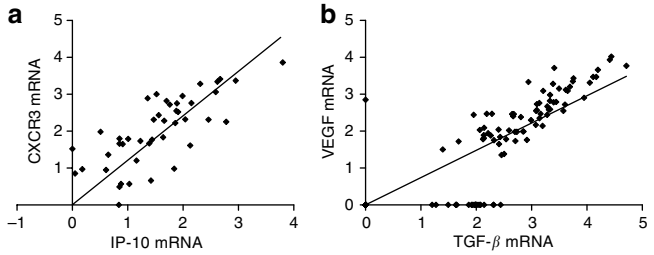

Figure 3 | Coordinated expression of mRNAs for chemokines and growth factors in urine cells. (**a**) The relationship between the levels of IP-10 and CXCR3 was significant at $P < 0.0001$ ($R = 0.78$). (**b**) The TGF-β and VEGF urinary mRNA was significantly correlated at $P < 0.0001$ ($R = 0.88$).

DISCUSSION

Successful management of LN requires a prompt diagnosis and early treatment. Abnormal urine sediments or proteinuria regardless of its degree or pattern (persistently or episodically) may be found in association with progressive renal pathology despite stable renal functions. Multiple urinary biomarkers have been shown to correlate with clinical criteria such as SLEDAI or clinically defined renal flare.[12,13,26] The present study verifies the potential utility of novel urinary biomarkers in patients who had pathologically confirmed proliferative (class IV) LN. Importantly, the application can be extended to the use in early prediction of therapeutic response in class IV LN patients. It should be emphasized that the class IV LN patients require potent immunosuppressive treatment. This monitoring tool may provide a better optimization of immunosuppressive agents in order to avoid their toxicities.

In this study, the measurement of urinary mRNA levels of IP-10 and CXCR3 chemokine genes, and TGF-β and VEGF growth factor genes is practical and should be useful in diagnosing class IV LN and in predicting clinical therapeutic response with high sensitivity and specificity. IP-10 appears to be the best candidate, followed by TGF-β, despite the fact that some overlapping levels of these mRNA can occur among patients with class IV and others, particularly class VI (diffuse glomerulosclerosis) (Figure 1). It should be noted that the two patients of class VI LN actually transformed from class IV LN and they showed resistance to prior cytotoxic therapy. Interestingly, the patients with class VI LN

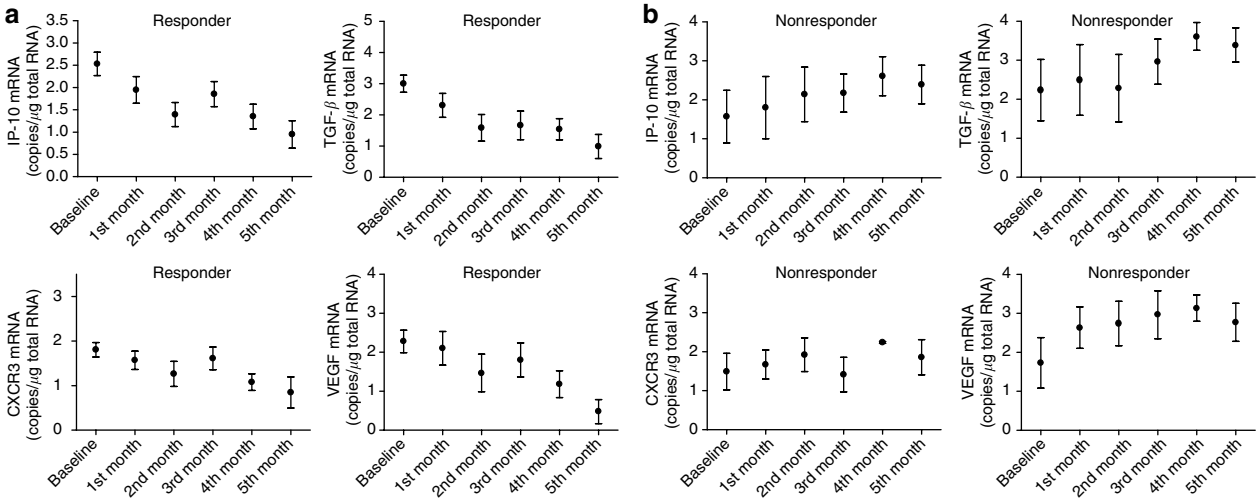

Figure 4 | Changes in urinary mRNA levels of IP-10, CXCR3, TGF-β, and VEGF over the 6-month period of treatment in patients with LN class IV. Dots and bars show means and s.e.m.'s of (log)mRNA levels for IP-10, CXCR3, TGF-β, and VEGF in urine samples from baseline through the fifth month of treatment. (**a**) The levels of IP-10, CXCR3, TGF-β, and VEGF were significantly decreased in the responder group, (**b**) whereas the levels of these genes were not changed in the nonresponder group.

had high urinary mRNA levels of TGF-β and VEGF, but not of IP-10 and CXCR3. The above findings suggest that the growth factors, not chemokines, reflect an ongoing process of tissue repair and fibrosis in the kidney. In contrast, the patient with class III (focal proliferative), which subsequently progressed into class IV, LN showed high urinary mRNA levels of both chemokines and growth factors. Taken together, monitoring of urine cells gene expression with combined biomarkers may serve as a reliable tool to distinguish class IV LN from other classes.

Currently, renal histopathologic study is the standard method of determining the presence of inflammatory process in nephritis patients. However, studying renal pathology may not give details on the progression of nephritis over a time span unless the hazardous renal biopsies are repeated. Monitoring gene expression in urine cells over time gives additional information, which would be complementary to the pathological study in understanding the ongoing inflammatory process of nephritis. Increased urinary mRNA levels of IP-10 and CXCR3 in only class IV LN support the role of Th-1 chemokines in intrarenal inflammation. Alternatively, the upregulation of TGF-β and VEGF in class IV and VI (diffuse glomerulosclerosis) may reflect tissue repair and fibrosis. It would be of interest to study whether molecular signaling could predict long-term renal function. Although the present cohort's follow-up period was short, we believe that the highly sustained expression of IP-10 and CXCR3 distinguishes an ongoing renal inflammation. In agreement with the study reported by Rovin *et al.*,[26] serial measurement of urine chemokine, monocyte chemoattractant protein-1 protein, predicts clinical renal flare and response to therapy in their prospective study. It remains intriguing as to which parameters could predict long-term renal function. Upregulation of TGF-β and VEGF found in class IV and VI in this report may reflect tissue repair and fibrosis. Alternatively, such persistent expression may predict poor outcome in the future, with sclerosis and fibrosis of the entire kidney.

Multiple conventional markers as shown here, such as proteinuria and SLEDAI, ineffectively discriminate class IV LN from others. Only urine erythrocyte count can reliably distinguish class IV renal pathology. Nevertheless, variable numbers of urine erythrocyte counts could be found in most female patients during each visit. Therefore, the presence of erythrocyte in urine cannot predict the response to treatment. There are pressing needs for a short-term LN treatment trial to compare the outcome, since current management relies on more rigid criteria, such as renal or patient survival, which require at least 5 years to complete. Given the key functions of the studied genes, the measurement of their gene transcripts may replace the conventional markers being used as surrogate markers in the clinical trials of molecularly targeted therapy such as cytokine or growth factor blockade.

Repeated attacks of LN, particularly diffuse nephritis, is the worst prognostic factor in the development of end-stage renal disease.[5] Most SLE patients, however, die from complications and infections partly from overuse of immunosuppressive agents. The monitoring of urine cell chemokines and growth factors described herein may be another useful approach for early detection of the critical stage of inflammation in the kidney of SLE patients and for tailoring immunosuppressive therapy.

MATERIALS AND METHODS

Patients

In total, 26 patients who had been diagnosed with SLE according to the American College of Rheumatology diagnostic criteria for SLE were recruited between August 2002 and September 2004. A total of 52 urine samples were obtained. Two urine samples were collected from each individual patient approximately 2 weeks and 1 day before renal biopsy. Renal involvement was documented by having one of the following criteria: a total urinary protein level of more than 0.5 g/day, an increment of serum creatinine levels of more than 0.5 mg/dl during 1 month period of follow-up, or presence of pyuria, hematuria, or urinary cast by microscopic examination. All biopsy specimens were examined by one pathologist (VK) who was not aware of the results of the molecular study. Using the classification of the histologic types of LN by World Health Organization,[27,28] 14 of 26 biopsy specimens were identified as class IV, whereas the remaining 12 specimens were categorized into other classes. All 26 patients received prednisolone (0.5 mg/kg/day) without other cytotoxic drugs, prior to renal biopsy for at least 1 month. Four patients received 5–10 mg per day of enalapril. In all, 10 urine samples from healthy women served as controls.

This study has been approved by the Ethics Committee for Human Research of the Faculty of Medicine, Chulalongkorn University, and written informed consents were obtained from all subjects.

Of the 26 patients, 14 patients with class IV LN were prospectively followed. These patients received oral prednisolone at the dosage of 0.5 mg/kg/day plus a 6-month regimen of either monthly intravenous pulse cyclophosphamide or oral mycophenolate mofetil. Urine samples were collected monthly from pretreatment baseline to the fifth month of treatment. Therapeutic response was defined either by the improvement of pathological scores of activity and chronicity based on repeated kidney biopsies, or by the following clinical criteria, including: (1) stabilization or improvement in renal function; (2) $\geqslant$50% decrease in hematuria to less than 10 RBC per high-power field; and (3) significant reduction in proteinuria ($\geqslant$50% decrease to less than 3 g/day if baseline nephrotic range, $\leqslant$1 g/day if baseline non-nephrotic) for at least 3 months.[29]

Collection of urine sample and RNA isolation

Urine was immediately centrifuged after collection at 1000 *g* for 30 min at 4°C. Total RNA was isolated from the cell pellets using an RNA blood mini kit (Qiagen, Chatworth, CA), measured for concentration and reverse-transcribed into complementary DNA as described previously.[21]

Analysis of urinary mRNAs

The mRNA levels of IP-10, CXCR3, TGF-β, VEGF, and the housekeeping gene, 18s rRNA, were measured by a Light Cycler machine (Roche Molecular Biochemicals, Indianapolis, IN). The sequences of primers and fluorescence probes are as follows: IP-10

sense 5′att ttg tcc acg tgt tga gat ca3′, IP-10 antisense 5′tgg cct tcg att ctg gat tc3′ and IP-10 probe 5′ 6-carboxy-fluorescein (FAM) aca tct ctt ctc acc ctt ctt ttt cat tgt agc a 6-carboxy-tetramethylrodamine (TAMRA)3′; CXCR3 sense 5′acc cag cag cca gag cac3′, CXCR3 antisense 5′caa cct cgg cgt cat tta gc3′, and CXCR3 probe 5′FAM ctt ggt ggt cac tca cct caa gga cca t TAMRA3′; TGF-β sense 5′ccc tgc ccc tac att tgg ag3′, TGF-β antisense 5′ ccg ggt tat gct ggt tgt aca3′, and TGF-β probe 5′FAM cac gca gta cag caa ggt cct ggc c TAMRA3′; VEGF sense 5′cct aca gca caa caa atg tga atg3′, VEGF antisense 5′ caa atg ctt tct ccg ctc tga3′, and VEGF probe 5′FAM caa gac aag aaa atc cct gtg ggc ct TAMRA; 18s rRNA sense 5′gcc cga agc gtt tac ttt ga3′, 18s rRNA antisense 5′tcc att att cct agc tgc ggt atc3′, and 18s rRNA probe 5′FAM aaa gca ggc ccg agc cgc c TAMRA3′. The probes were labeled with FAM at the 5′ end, and with TAMRA at the 3′ end. FAM serves as the reporter dye, and TAMRA serves as the quencher dye. All primer pairs were designed to span across an intron–exon boundary to distinguish an amplification of genomic DNA. Each polymerase chain reaction was set up for 20 μl reaction volume comprising of 18 μl of polymerase chain reaction mastermix (Roche Molecular Biochemical, Mannheim, Germany), 2 μl of complementary DNA template (diluted 1:1000 for 18s rRNA), 600 nM of primer, and 200 nM of probe. Polymerase chain reaction amplification included an initial denature at 95°C for 2 min, followed by heating at 95°C for 10 s and 60°C for 10 s repeated for 40 cycles. The TGF-β and VEGF plasmids (kindly provided by Karumanchi SA, Harvard Medical School, Boston, MA) and polymerase chain reaction amplicon for 18s rRNA (kindly provided by Ding R, Cornell University, New York, NY) were used for developing standard curves. The standard curves were based on the principle that a plot of the log of the initial target copy of a standard vs threshold cycles results in a straight line. Levels of mRNA were expressed as number of copies per microgram of total RNA isolated from the urine cells.

Kidney biopsy

Kidney biopsy specimens were scored for activity and chronicity index. The activity index was calculated from 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 were counted double, yielding the total range for the activity index of 0–24. The chronicity index was the sum of scores of the following parameters: glomerular sclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis.[30] The maximum score of the chronicity index was 12.

Statistical analysis

Statistical analysis was performed using the SPSS software (version 11.5). The levels of all studied mRNAs deviated significantly from normal distribution ($P<0.001$), which was substantially reduced by the use of a log transformation. The natural log mRNA levels were used as dependent variables in comparison for any variable difference by Student's *t*-test (between two groups) and one-way analysis of variance with Bonferrini's correction (between groups). The relationship between the mRNA levels of each gene was estimated with Pearson's correlation. To distinguish class IV LN from others, receiver operator characteristic curve analysis of mRNA levels was used to determine the cutoff levels that maximized the combined sensitivity and specificity. The area under the curve was calculated, and sensitivity and specificity at the selected cutoffs were determined.

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