Research Article

Urine Angiostatin, CXCL4 and VCAM1 as predictors of renal versus non-renal activity in Lupus

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Abstract
Objective: To study urinary angiostatin, CXCL4 and VCAM-1 as biomarkers of renal disease in systemic lupus erythematosus (SLE). **Methods:** Patients who fulfilled ≥4 ACR criteria for SLE with active renal, active non-renal or inactive disease and a group of healthy controls were studied. Urine samples were assayed for angiostatin, CXCL4 and VCAM-1 by ELISA, and normalized by creatinine. Receiver operating characteristic (ROC) analysis was performed to obtain the best cut-off values to calculate the performance of these markers in differentiating among the different groups of patients as compared to anti-dsDNA and complement C3. Correlation between these urinary biomarkers with various renal parameters was also performed. **Results:** 175 SLE patients (56 inactive SLE; 65 active non-renal disease; 54 active renal disease; (94.3% women, mean age 34.7±12.9 years) and 53 controls (96% women) were studied. Urinary angiostatin, CXCL4 and VCAM-1 levels (normalized for creatinine) were significantly higher in patients with active renal than active non-renal disease, inactive SLE or controls. These markers correlated significantly with the total SLE disease activity index (SLEDAI) and renal SLEDAI scores, as well as the urinary protein-to-creatinine ratio. Angiostatin had the highest specificity and sensitivity in differentiating active renal from active non-renal SLE (area under the curve [AUC] 0.87). CXCL4 (AUC 0.64) and VCAM-1 (AUC 0.73), on the other hand, performed similarly to anti-dsDNA or C3. **Conclusions:** Urinary angiostatin, CXCL4 and VCAM-1 are potential useful biomarkers for SLE, in particular lupus nephritis. Further longitudinal studies are necessary to delineate the performance of these markers in predicting renal flares and prognosis in SLE patients.
Key words: biomarker, lupus, nephritis, adhesion molecule, anti-angiogenic, chemokine

Introduction
SLE is a complex systemic autoimmune disease with heterogeneous manifestations, with Lupus nephritis (LN) remaining a major cause of morbidity and mortality among patients. Overt renal disease is found in 15-30% of patients with lupus at the time of initial diagnosis and 30-50 % during disease progression, with reports of 5-year renal survival rate reaching 46-95% [(1-3)]. Present assessment of LN includes serologic measurement of anti-ds DNA and complement levels. These although being clinically valuable methods, they are neither specific to renal involvement nor predictive of the classification and severity of renal pathology with biopsy[(4)].

Till now renal biopsy remains the gold standard for diagnosis and assessing prognosis of LN, however, its invasiveness and hazardous complications warrant the discovery of novel noninvasive diagnostic and prognostic biomarkers[(5)]. Over the past decade, there has been huge interest in defining reliable lupus nephritis biomarker for assessing disease activity and progression; however, no candidate bio-marker has yet been validated[(6)]. Urinary biomarkers are considered attractive candidates for reflecting the activity of LN as they may be the most immediate and excellent indicators of change in the renal status. Among the potential biomarkers, Angiostatin and VCAM-1 have emerged and proven effective in tracking disease activity in LN.

Proteomics study is now considered as an efficient screening approach for identifying valuable protein biomarkers which are worth tracing. A preliminary proteomic

study revealed increased levels of urinary Angiostatin in SLE patients especially patients with class IV LN, showing the highest levels, in addition to its ability to differentiate active SLE from inactive SLE and its significant correlation with renal SLICC score as well as renal pathology chronicity index[7]. Similarly, Vascular Cell Adhesion Molecule-1 (VCAM-1) has been found to be elevated in the kidney, serum and urine of SLE patients generally and LN specifically with strong association with renal pathology activity index, physician's global estimate of disease activity and SLICC renal activity score[8,9]. Angiostatin, the N-terminal fragment of plasminogen, was originally purified as a potent angiogenic inhibitor in mice with Lewis lung carcinoma. It specifically inhibits proliferation and induces apoptosis in vascular endothelial cells, thus inhibiting tumor growth. The anti-inflammatory properties of angiostatin were recently reported. VCAM-1, a member of the immunoglobulin superfamily, is involved in recruitment of inflammatory cells via interaction with an integrin located on leukocytes.

CXCL4, also known as Platelet Factor 4 (PF4), is found to be the most predominant protein biomarker in Systemic sclerosis. A proteome-wide analysis and validation showed CXCL4 to be significantly higher in patients with systemic sclerosis compared to healthy controls. It also correlated significantly with the development and progression of complications such as lung fibrosis and pulmonary arterial hypertension[10]. In view of the paucity of data of these three urinary protein markers in lupus nephritis, especially CXCL4, we conducted this cross-sectional study to evaluate the performance of these markers in predicting active renal disease in SLE, as compared to conventional SLE markers.

Methods

Study population

Adult patients (≥18 years of age) who diagnosed as SLE according to the 1997 American College of Rheumatology (ACR) classification criteria[11] were recruited into our study. Urine samples were collected for the assay of the three biomarkers studied, namely angiostatin, CXCL4 and VCAM-1. A group of healthy subjects were also recruited as controls. Written informed consent was obtained from the participants and this study was approved by the Ethics Committee of the hospital administration.

Patients recruited were stratified into 3 groups: clinically inactive SLE, active non-renal SLE and active renal SLE. A fourth group was healthy controls. Clinical data that include demographic and clinical characteristics, renal parameters (urine protein-to-creatinine ratio and sediments, and serum creatinine for those with active renal disease) were collected at the time of recruitment. SLE disease activity and organ damage was assessed. Urinary protein marker levels were compared in these patient groups and controls. Correlation between the urinary markers and various renal parameters was also performed.

Assessment of disease activity and organ damage

SLE disease activity was assessed by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), which is a validated tool to assess lupus activity in clinical and research basis[12]. Clinically inactive SLE was defined as a clinical SLEDAI score of zero.

Assay of the urinary protein markers

Urinary levels of angiostatin, CXCL4 and VCAM1 were assayed using enzyme-linked immunosorbent assay (ELISA). In particular, CXCL4 (Cat #: DY795) and VCAM1 (Cat #: DY809) were assayed using ELISA kits from R&D Systems (Minneapolis, Minnesota, USA), whereas angiostatin was assayed using an ELISA kit (Cat#: ELH-Angiostatin) from Raybiotech, Inc (Norcross, Georgia, USA). Urine samples were diluted 1: 5 for CXCL4, 1: 100 for VCAM1 and 1: 2 for angiostatin. Optical densities at 450nm were measured using a microplate reader ELX808 (BioTek Instruments, Winooski, VT) and sample concentrations were calculated using a standard curve. All measurements were assayed in duplicate. The values of these urinary protein markers were normalized to urine creatinine.

Statistical analyses

Unless otherwise stated, values in this study were expressed as mean ± standard deviation (SD). Comparison of values among different groups of subjects was performed by the non-parametric Kruskal-Wallis H (continuous variables) and the Chi-square tests (categorical variables). Correlation analysis between two variables was performed using the Spearman's rank correlation. Receiver Operating Characteristic (ROC) curve analysis was employed to study the best cut-off values of the protein markers to differentiate between active renal and non-renal SLE and between active and inactive SLE. The area under the curve (AUC) was calculated and the best trade-off point of sensitivity and specificity was determined from the values calculated for each of the coordinates on the curve. Elevation of the protein markers was defined by using the best cut-off values obtained from ROC analyses. Statistical significance was defined as a P value of less than 0.05, two-tailed. All statistical analysis was performed using SPSS (version 16.0, Chicago, IL).

Results

Study population

A total of 175 SLE patients (94.3% women) were studied. The mean age was 34.7±12.9 years and SLE duration mean was 9.1±7.1 years. 54 (31%) patients had active renal SLE, 65 (37%) had active non-renal SLE and 56 (32%) had clinically inactive SLE. Fifty-three healthy subjects (96% women; mean age 32.5±3.9 years) were recruited as controls.

Table 1 shows the clinical characteristics of the SLE patients studied. The total SLEDAI score were significantly higher in patients with active renal than non-renal SLE and inactive SLE. Mycophenolate mofetil was more frequently used in patients with active renal SLE, whereas hydroxychloroquine was more often used in patients with active non-renal SLE. The SLICC organ damage scores, however, were similar among the 3 groups of SLE patients.

Urine levels of angiostatin, CXCL4 and VCAM-1

Figure 1 shows the urine levels of angiostatin, CXCL4 and VCAM-1 in the 4 groups of subjects studied. Levels of all these 3 protein markers were significantly higher in patients with active renal disease than active non-renal disease, inactive SLE or healthy controls.

ROC curve analyses were performed for the AUCs and best cut-off values of these protein markers to differentiate between active renal and non-renal SLE and between active SLE and inactive SLE. The AUCs values are shown in Table 2. Among the three urine protein markers, angiostatin exhibited the highest AUC and specificity / sensitivity in differentiating active renal from active non-renal SLE, as well as, predicting active SLE from inactive SLE. CXCL4 and VCAM-1, on the other hand, had similar AUC, specificity / sensitivity to conventional serological markers (anti-dsDNA and complement C3) in distinguishing between active renal and non-renal SLE.

Correlation of the urine protein markers with SLE activity and renal parameters

Among patients with SLE (N=175), the three urine protein markers correlated significantly with the total SLEDAI (angiostatin: R 0.60, $p<0.001$; CXCL4: R 0.46, $p<0.001$; VCAM-1: R 0.53, $p<0.001$), renal SLEDAI (angiostatin: R 0.66, $p<0.001$; CXCL4: R 0.45, $p<0.001$; VCAM-1: R 0.51, $p<0.001$). In the active renal SLE group, these markers also correlated significantly with the urine protein-to-creatinine ratio (uPCR) (angiostatin: R 0.73, $p<0.001$; CXCL4: R 0.51, $p<0.001$; VCAM-1: R 0.59, $p<0.001$).

Table 1: Clinical characteristics of the studied SLE patients:

	Inactive SLE (N=56)	Active non-renal SLE (N=65)	Active renal SLE (N=54)	Total (N=175)	P¥
	Mean±SD; N (%)				
Age, years	36.3 ± 12.0	34.5 ± 13.6	33.3± 9.7	34.7± 12.9	0.752
Women	54 (96.4)	60 (92.3)	51 (94.4)	165 (94.3)	0.721
SLE duration, years	13.6± 6.1	4.1± 5.2	10.5± 6.4	9.1± 7.1	< 0.001*
Clinical disease activity					
Neuropsychiatric	-	7 (11)	4 (7.4)	11 (6.3)	0.14
Musculoskeletal	-	27 (41.5)	19 (35.2)	46 (26.3)	0.29
Renal	-	0 (0)	54 (100)	54 (31)	<0.001*
Mucocutaneous	-	35 (54)	18 (33)	53(30.3)	0.006*
Serositis	-	14 (21.5)	10 (18.5)	24(14)	0.46
Hematological	-	37 (57)	12 (22)	49 (28)	<0.001*
Total SLEDAI score$	2 (0-4)	9 (0-23)	20 (4-22)	8 (0-23)	<0.001*
SLICC damage score	0 (0-6)	0 (0-4)	0 (0-5)	0 (0-6)	0.97
Medications ever used					
Prednisolone	50 (89.3)	60 (92.3)	53 (98)	163 (93)	0.174
Azathioprine	41 (73.2)	39 (60)	4 (7.4)	84 (48)	< 0.001*
Cyclophosphamide	13 (23)	16 (25)	3 (5.5)	32 (18.3)	0.007*
Cyclosporine A	7 (12.5)	10 (15.4)	2 (3.7)	19 (10.9)	0.058
Tacrolimus	5 (9)	6 (9.2)	2 (3.7)	13 (7.4)	0.372
Mycophenolate mofetil	10 (18)	14 (21.5)	37 (68.5)	61 (34.9)	< 0.001*
Hydroxychloroquine	41 (73.2)	58 (89.2)	45 (83.3)	144 (82.3)	0.069

By (x^2) test and Kruskal-Wallis test

SLE = systemic lupus erythematosus; SD = standard deviation; SLEDAI = SLE disease activity index; SLICC = SLE international collaborative clinic; ¥: Comparison among the three groups; $: Median (IQR); *: Significant

Table 2: Performance and ROC analysis of the protein biomarkers in predicting disease activity among the studied patients:

SLE activity¥				
Marker	**AUC (95% CI)**	**Sensitivity (%)**	**Specificity (%)**	**P- value**
Angiostatin	0.84 (0.78- 0.9)	91	65	<0.0001*
CXCL4	0.76 (0.69-0.83)	92.9	57	<0.0001*
VCAM1	0.77 (0.7- 0.84)	89	61	<0.0001*
Anti-dsDNA	0.73 (0.65- 0.8)	96	50.5	<0.0001*
C3	0.66 (0.58-0.75)	67	62	0.0003*
C4	0.67 (0.58-0.76)	61	70.5	0.0002*
Lupus nephritis activity§				
Marker	**AUC (95% CI)**	**Sensitivity (%)**	**Specificity (%)**	**P- value**
Angiostatin	0.86 (0.8-0.9)	83	77.7	<0.0001*
CXCL4	0.65 (0.55- 0.75)	66	63	0.005*
VCAM1	0.61 (0.5- 0.7)	35	100	0.054
Anti-dsDNA	0.65 (0.55- 0.73)	63	66.6	0.004*
C3	0.59 (0.5- 0.7)	52	65	0.07
C4	0.53 (0.43- 0.64)	15	97	0.53

ROC = receiver operating characteristic curve; AUC = area under the curve; SLE = systemic lupus erythematosus; CI = confidence interval; ¥: Active SLE Vs. Inactive SLE; §: Active Renal SLE Vs. Active Non-renal SLE; *: Significant

Figure 1: Urine levels of angiostatin, CXCL4 and VCAM-1 among all subjects:

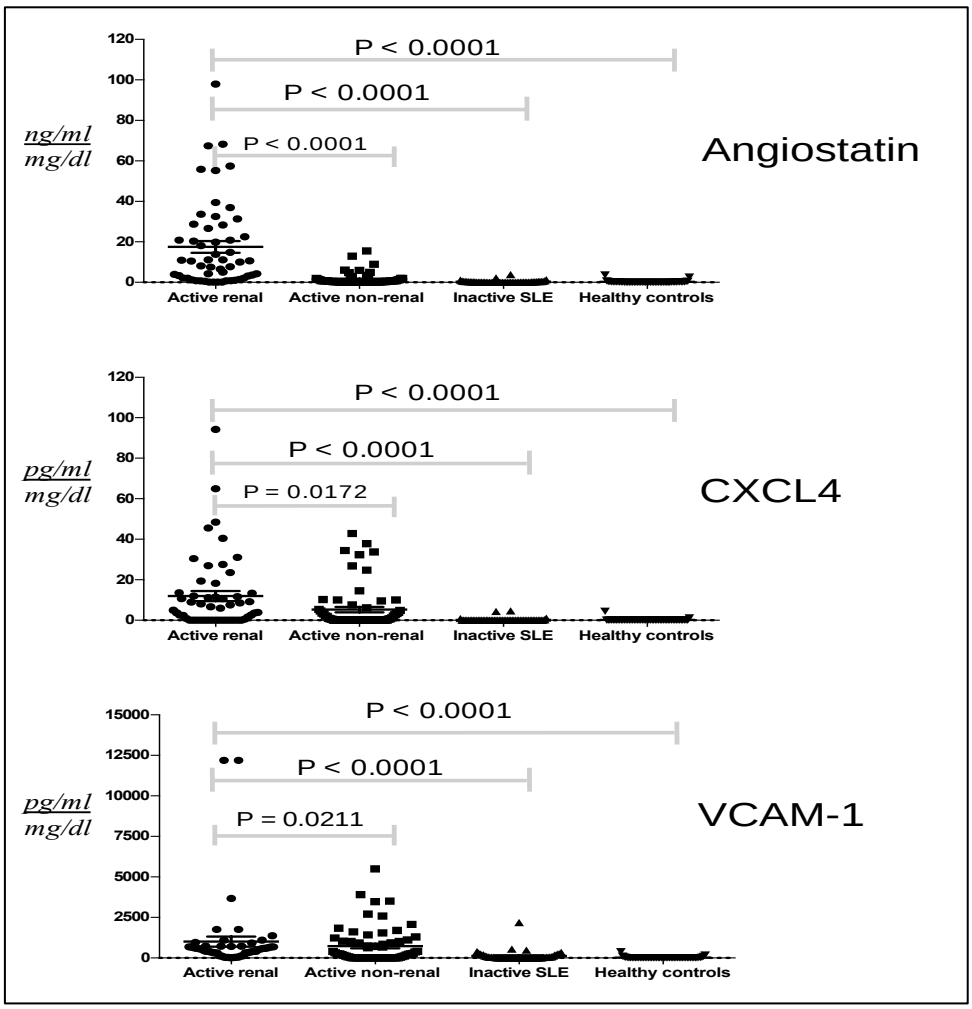

Figure 1: Urinary levels of angiostatin, CXCL4 and VCAM-1 in the 4 groups of subjects studied; Urinary biomarker levels were normalized to urinary creatinine. The Y axis shows the values of the 3 studied biomarkers, while the X axis has the 4 studied groups (Active renal, avtive non-renal, Inactive SLE and healthy controls). Non-parametric Kruskal-Wallis test was used to test the difference among the 4 groups of patients.

Discussion

In this cross-sectional study, we showed that the urinary levels of angiostatin, CXCL4 and VCAM-1 were significantly higher in patients with active renal SLE than active non-renal or inactive SLE. The urinary levels of these markers correlated significantly with the SLE disease activity score, renal activity scores and the urinary protein levels. These markers have the ability to differentiate active renal from active non-renal SLE, as well as active from inactive SLE. The overall results suggested that these urinary proteins are potential biomarkers in patients with SLE, particularly those with LN.

Angiostatin, the N-terminal fragment of plasminogen, is a potent angiogenesis inhibitor that has been shown to mediate suppression of metastases by a Lewis lung carcinoma. In a mouse model of chronic kidney injury, treatment with recombinant adeno-associated viruses expressing angio-statin retarded the progression of kidney disease, likely due to the anti-inflammatory actions of this anti-angiogenic protein[13]. In a previous study of African American,

Hispanic and Caucasian patients with SLE, urinary angiostatin was increased in active SLE, particularly active LN[7]. The results of the present study, which involved a larger group of patients with SLE, confirmed the finding that urinary angiostatin was a marker that could differentiate active renal from active non-renal SLE with a higher specificity / sensitivity than anti-dsDNA and complement C3.

Soluble VCAM-1 levels are elevated in several autoimmune diseases that include SLE and rheumatoid arthritis[14,15]. Previous studies have demonstrated that urinary VCAM-1 was elevated in patients with active SLE or LN[8,9,16]. A recent study has previously shown that urinary VCAM-1 level was increased in various ethnic patients with LN, and correlated with SLEDAI scores and histologic renal activity[8]. This is consistent with the current study that urinary VCAM-1 levels were elevated in active LN, and could differentiate active renal from non-renal disease. As urine VCAM-1 did not correlate with the degree of proteinuria, it should be further explored as a urinary marker that may predict flares of LN independent of proteinuria.

Like angiostatin, CXCL4 is another potent anti-angiogenic chemokine that influences angiogenesis by an integrin-dependent mechanism[17]. Circulating CXCL4 levels were increased in patients with systemic sclerosis and correlated with progression of heart and lung disease[10]. Because the platelet is the main source of circulating CXCL4, this chemokine is postulated to be associated with atherosclerosis and thrombosis[18,19]. However, the origin and mechanism of CXCL4 excretion in the urine in patients with immune-mediated glomerulonephritis remains unclear. A study of patients with subclinical tubulitis, which was associated with the development of chronic kidney tubular lesions, did not report an elevation of urinary CXCL4 levels[20]. In the current study, we showed that urinary CXCL4 was elevated in patients with active SLE and LN. CXCL4 should further be evaluated as a potential biomarker for LN flares and prognosis (renal fibrosis) in long-term longitudinal studies.

There are a couple of limitations in the current study. First, the design is cross-sectional. Although we showed that these novel urinary markers correlated with SLE renal activity and were able to differentiate active renal from non-renal SLE, their role in predicting flares of lupus nephritis is still unclear. This should be addressed by long-term longitudinal studies. Second, because of the cross-sectional design, we did not have data on the predictive values of these urinary markers on the progression of LN. Despite these caveats, our study has provided further evidence to suggest a potential role of urinary angiostatin, CXCL4 and CVAM-1 as predictors of renal involvement in patients with SLE. Further longitudinal prospective studies will provide more information on the performance of these urinary protein markers in predicting flares and prognosis of LN as compared to conventional markers and urinary protein quantification.

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Conflict of interest: The authors declare that there is no conflict of interest.

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