Messenger RNA expression of target genes in the urinary sediment of patients with chronic kidney diseases

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Abstract

Background. The degree of renal scarring in kidney biopsy is an important prognostic factor in patients with chronic kidney diseases. We hypothesize that gene expression in the urinary sediment reflects the degree of renal damage.

Methods. We studied 29 patients with chronic kidney disease who underwent kidney biopsy (12 immunoglobulin-A nephropathy and 17 glomerulosclerosis) and 10 healthy controls. The mRNA expressions of a panel of target genes in urinary sediment were measured by real-time quantitative polymerase chain reaction. The results were compared with the degree of histological damage and renal function decline.

Results. There were significant differences in the urinary expression of transforming growth factor-β (TGF-β), monocyte chemotactic protein-1 (MCP-1) and collagen IV between disease groups and controls. Urinary TGF-β mRNA expression correlated significantly with estimated glomerular filtration rate ($r = -0.412, P = 0.029$) and the degree of tubulointerstitial scarring ($r = 0.418, P = 0.024$). Urinary MCP-1 expression correlated with the degree of glomerulosclerosis ($r = 0.450, P = 0.014$), but not tubulointerstitial scarring. Urinary MCP-1 expression correlated with its corresponding level by enzyme-linked immunosorbent assay (ELISA) ($r = 0.650, P < 0.001$), but TGF-β expression did not correlate with its ELISA level. Urinary TGF-β gene expression correlated with its intra-renal expression in glomeruli ($r = 0.701, P < 0.001$) and tubulointerstitium ($r = 0.573, P < 0.001$) by immunohistochemistry, while urinary MCP-1 gene expression correlated with its staining in glomeruli ($r = 0.576, P = 0.001$) but not tubulointerstitium. After 12 months, there was a significant inverse correlation between the rate of renal function decline and urinary expression of connective tissue growth factor ($r = -0.471, P = 0.010$) and collagen I ($r = -0.399, P = 0.032$), but not TGF-β or MCP-1.

Conclusions. Amongst the target genes examined, the mRNA expression of TGF-β in urinary sediment correlated with renal function, the degree of histological damage and intra-renal level in patients with chronic kidney diseases. Measurement of TGF-β mRNA expression in urine may be a useful non-invasive tool for assessing the severity of renal damage in patients with chronic kidney diseases.

Keywords: monocyte chemotactic protein; renal failure; transforming growth factor

Introduction

Chronic kidney disease is a debilitating and costly medical condition. The clinical course is characterized by persistent proteinuria after an initial insult to the kidney, followed by progressive decline in renal function [1]. Proteinuria and the severity of scarring in kidney biopsy are the most reliable prognostic indicators in patients with chronic kidney disease [1–3]. However, histological damage develops late in the pathogenic pathway and kidney biopsy is an invasive test. Since tubulointerstitial inflammation may precede renal scarring, measurement of cytokines in urine is the logical means to assess the severity of kidney damage and provide prognostic information.

Many previous reports have indicated significant correlation between urinary levels of various cytokines and the severity in many types of progressive renal diseases [1,4–7]. However, measurement of urinary cytokine protein may not be clinically relevant. Most of the cytokines exert paracrine effects and are not excreted in the urine. In fact, renal tubular cells are
organized into highly specialized luminal and basolateral compartments. Therefore, the amount of cytokine protein found in urine may not correlate with the local concentration in renal interstitium [8,9].

We hypothesize that measurement of gene expression in urinary sediment at the messenger RNA (mRNA) level would be the logical non-invasive surrogate marker of renal inflammation and fibrosis. Quantification of gene expression in urinary sediment at the mRNA level has become possible recently [10,11]. Extraction of mRNA from urinary sediment is an established technique [10] and mRNA expression of cytokines can be examined by the reverse transcription and real-time quantitative polymerase chain reaction assay (RT–QPCR). A recent study reports that mRNA expression of granzyme in urinary sediment correlates with the severity of kidney allograft rejection [10]. Recently, we have demonstrated a predominance of T-helper cell type 1 pattern of cytokine mRNA expression in the urinary sediment of patients with active lupus nephritis [11]. In the present study, we examined the expression of a panel of target genes in the urinary sediment of patients with chronic kidney diseases to explore the inter-relationship between urinary gene expression and the severity of renal damage in patients with chronic kidney diseases.

Subjects and methods

Patients

We studied 29 consecutive patients who required kidney biopsy for chronic kidney diseases with proteinuria. In 12 of them, kidney biopsy confirmed primary immunoglobulin-A (IgA) nephropathy (the IgAN Group), which included grade I, II and III disease (four cases each) according to the conventional histological grading of IgA nephropathy described previously [12]. In the other 17 patients, kidney biopsy showed glomerulosclerosis, which included hypertensive nephrosclerosis (13 cases) and focal glomerulosclerosis (four cases); they were designated as the Sclerosis Group. In all patients, serum creatinine, urea and albumin were measured by conventional methods within 1 month prior to kidney biopsy. Glomerular filtration rate (GFR) was estimated by a standard equation [13]. Proteinuria was determined by 24 h urine collection.

Quantification of urinary mRNA expression

A whole-stream early morning urine specimen was collected on the morning of kidney biopsy after informed consent. Urinary sediment isolation and mRNA extraction were performed as described by Li et al. [10] and modified by our group [11]. Briefly, the urine specimen was centrifuged at 3000 g for 30 min at 4 °C. After centrifugation, examination of the urinary sediment with phase-contrast microscopy was performed by a single observer (R.W.Y.C.) and cell viability was checked by trypan blue staining. Based on our previous data [11], centrifugation at a speed >3000 g did not affect the quantity or quality of mRNA being extracted. The supernatant was stored at −70 °C until the enzyme-linked immunosorbent assay (ELISA) study of urinary cytokine (see below). Total RNA was extracted from the centrifuged sediment by the RNeasy Mini Kit (Qiagen Inc., Mississauga, ON, Canada), following the manufacturer’s instructions. All specimens were pre-treated with deoxyribonuclease I (Invitrogen™; Life Technologies, Philadelphia, USA) and then stored at −70 °C until use. The purity of RNA was confirmed by the relative absorbance at 260/280 nm ratio using a spectrometer. The integrity of RNA isolated from urinary sediment by this method has previously been shown to be adequate for RT–QPCR [10]. This is also demonstrated in our subjects by running total RNA in denaturing 1.5% agarose gel, which demonstrated clear 28S and 18S ribosomal RNA (rRNA) bands (Figure 1).

We used 0.5 μg RNA for reverse transcription with the Superscript II RNase H Reverse Transcriptase (Invitrogen™; Life Technologies, Philadelphia, USA). The mRNA expressions of cytokines were quantified by RT–QPCR with the use of an ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The targets we studied included monocyte chemotactic protein-1 (MCP-1) (a major chemokine), transforming growth factor-beta (TGF-β), connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), collagens I, III and IV and fibronectin (markers of tubular scarring), alpha-smooth muscle actin (marker of
epithelial–mesenchymal transdifferentiation of myofibroblast) and caspase-3 (marker of tubular apoptosis). RT-QPCR of CTGF, fibronectin, caspase-3 and collagens I, III and IV was performed using commercial kits (all from Applied Biosystems), following the manufacturer’s instructions. We used commercially available Taqman primers and probes for all the other targets (all from Applied Biosystems). The primer and probe sequences of MCP-1 were: forwards, 5'-TTC TTC CAC CAT GCC GCAG-3'; reverse, 5'-CCA GGC AAC TGT GA-3'; probe, 5'-CCC TGT CAT GCT TCT GGG CCT GC-3'. The primer and probe sequences of TGF-β were: forwards, 5'-CCC AGT ATC TGC AAA GCTC-3'; reverse, 5'-GTC AAT GTA GAC CTT CCG CA-3'; probe, 5'-ACA CCA ACT ATT GCT TCA GCT CCA CGGA-3'. The primer and probe sequences of VEGF were: forwards, 5'-TAC TTC CAC CAT GCC AAG TG-3'; reverse, 5'-CTG CAG CAG GAA GAT GTC CAC CA-3'; probe, 5'-TCA GGG CAG CTA CTG CCA TCC AAT-3'. The primer and probe sequences of HGF were: forwards, 5'-TGTT TTC ACA AGT CAA GAC GAG GTA-3'; reverse, 5'-CAG GTC ATG CAT TCA ACT TCT GA-3'; probe, 5'-AAAT GTC ACA GAC TTCG-3'. The primer and probe sequences of alpha-smooth muscle actin were: forwards, 5'-GAC GAA GCA CAG AGC AAA AGAG-3'; reverse, 5'-TGG TGA TGA TGC CAT GTT CTA TCG-3'; probe, 5'-CTT GAC CCT GAA GTA CC-3'. The level of mRNA expression of each target was normalized to 18s rRNA as the house-keeping gene (Applied Biosystems). RT-QPCR amplifications were performed in a 20 µl volume at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All tests were performed in triplicate. Results were analysed with Sequence Detection Software version 1.9 (Applied Biosystems).

Gene expression for each signal was calculated by using the difference-in-threshold-cycle (ΔC_t) procedure, according to manufacturer’s instructions. For 18s rRNA and each target, the relative efficiency of amplification over various starting template concentrations was determined. Approximately equal efficiencies for other targets with 18s rRNA amplifications were verified by an absolute value of less than 0.1 for the slope of log input cDNA amount vs ΔC_t, which was obtained by subtracting the threshold cycle (C_t) value of 18s rRNA from that of the target. Therefore, it was possible to detect 18s rRNA in the same tube with other targets. The relative quantification of using multiplex reaction with a comparative method was determined by the formula: 2^(-ΔΔC_t), where the ΔΔC_t was calculated by the subtraction of ΔC_t of calibrator from ΔC_t of sample [14]. We studied 10 healthy volunteers as control. The expression level of target was a ratio relative to that of the healthy volunteers.

**Urinary cytokine ELISA**

Urinary MCP-1 and TGF-β concentrations at the protein level were determined by using the human MCP-1 and TGF-β ELISA kit (Quantikine®, R&D Systems, Abingdon, UK). The assays were performed according to the manufacturer’s instructions. To activate latent TGF-β, 0.5 ml urinary supernatant was incubated with 0.1 ml 1 N HCl. After mixing and incubation for 10 min at room temperature, the acidified samples were neutralized by adding 0.1 ml 1.2 M NaOH/0.5 M HEPES and then the sample was assayed immediately. The enzymatic reaction was detected at 450 nm with reference wavelength at 570 nm in an automatic microplate reader (Spectrafluorplus; Tecan, Collingwood, Australia). The detection limits of the ELISA kits were 5 and 7 pg/ml for MCP-1 and TGF-β, respectively. All tests were performed in duplicate. Urine MCP-1 and TGF-β levels were standardized by the amount of creatinine in the urine and expressed as pg/mg of creatinine.

**Morphometric study of kidney biopsy**

Sections (5 µm thick) of renal biopsy specimens were stained with Jones’ silver stain. Morphometry analysis of renal scarring was performed using computerized image analysis as described by Bruneval et al. [15]. Briefly, a Leica TWIN Pro image analysis system (Leica Microsystems, Wetzlar, Germany) was connected to a Leica DC500 digital camera on a Leica DMRXA2 microscope working with a 40× objective (final calibration: 0.258 µm/pixel) and to a microcomputer for storage of the morphometric measurements and to perform image analysis by using image-analysing software (MetaMorph 4.0; Universal Imaging Corporation TM, Downingtown, PA, USA). Ten glomeruli and 10 randomly selected areas were assessed in each patient and the average percentage of scarred glomerular and tubulointerstitial areas, as represented by the percentage of the area with positive staining, were computed for each patient.

**Study of intra-renal cytokine level**

The intra-renal levels of TGF-β and MCP-1 were determined by immunohistochemistry on the paraflin-embedded tissues (2 µm thick) from kidney biopsy of 23 patients in the Active Group with the conventional method. Briefly, the tissues were blocked with 0.3% H2O2 in methanol for 10 min at room temperature and 3% normal serum for 30 min at 37°C after deparaffinization. Sections were then incubated with the primary monoclonal mouse anti-human TGF-β antibody (Chemicon, York, UK) or MCP-1 antibody (Peprotech, London UK) in a dilution of 1:300 at room temperature overnight. After addition of the secondary antibody (dilution 1:100, peroxidase-conjugated goat anti-mouse immunoglobulins; DAKO, Copenaghen, Denmark), the sections were incubated with streptavidin (dilution 1:100; DAKO, Denmark), developed in diaminobenzidine and counterstained with haematoxylin. The intensity of TGF-β and MCP-1 staining in glomeruli and tubulointerstitial areas, as represented by the percentage of the area with positive staining, were quantified by morphometric analysis as described above.

**Clinical follow-up**

After kidney biopsy, all patients were followed for 12 months. Clinical management was by individual nephrologist and not affected by the study. Renal function test, including serum creatinine, urea and albumin levels, was assessed at least every 6 months. As mentioned above, estimated GFR was calculated by a standard equation [13]. The rate of GFR decline was calculated by the least-square regression method.

**Statistical analysis**

Statistical analysis was performed by SPSS for Windows software version 10.0 (SPSS Inc., Chicago, IL, USA). The results are presented as means ± SD unless otherwise
specified. Because the data were highly skewed, mRNA expression and cytokine levels by ELISA were compared between groups by the Mann–Whitney U-test. Correlations between mRNA expression or protein levels and the clinical or histological parameters were determined by Spearman’s rank correlation coefficient. A P-value of <0.05 was considered as statistically significant. All probabilities were two-tailed.

Results

The baseline demographic and clinical data of the 29 patients are summarized in Table 1. There was no significant difference in baseline renal function between the IgAN and Sclerosis Groups. Although the Sclerosis Group had marginally more severe glomerulosclerosis and tubulointerstitial scarring than the IgAN Group, the result was not statistically significant.

Urinary mRNA expression between disease groups and controls

We first compared the levels of gene expression between disease groups and controls. There were significant differences in the urinary expression of TGF-β, MCP-1 and collagen IV and marginal differences in the expression of CTGF, HGF and collagen III between diagnosis groups (Figure 2). Post hoc analysis showed that the Sclerosis Group had significantly higher urinary expression of TGF-β and MCP-1 than the Control Group and both Sclerosis and IgAN Groups had higher collagen IV expression than the Control Group. In addition, the Sclerosis Group had significantly higher TGF-β, and lower HGF, expression than the IgAN Group.

Urinary mRNA expression and clinical parameters

The correlations between estimated GFR, proteinuria, degree of glomerular and tubulointerstitial scarring, rate of decline of renal function and the expression

![Figure 2](image_url)

**Fig. 2.** Comparison of gene expression in urinary sediment between diagnosis groups. Data are compared by one-way analysis of variance. Gene expressions are expressed as the ratio to the average expression of the control group. Error bars denote SDs. CTL, Control Group; Scl, Sclerosis Group; IgAN, IgA nephropathy Group; FN, fibronectin; ASMA, alpha-smooth muscle actin; COL, collagen.
of target genes in the urinary sediment are summarized in Table 2. The degree of proteinuria did not correlate with any of the target gene expression. There were significant correlations between estimated GFR and urinary expression of TGF-β (r = −0.412, P = 0.029) (Figure 3) but not other target genes. There were significant internal correlations between the target genes. For example, TGF-β expression correlated with that of MCP-1 (r = 0.332, P = 0.047).

The mRNA expression of several target genes in urinary sediment also correlated with the degree of histological damage in kidney biopsy (Table 2). For example, the degree of tubulointerstitial scarring significantly correlated with the expressions of TGF-β (r = 0.418, P = 0.024), CTGF (r = 0.541, P = 0.002) and collagen IV (r = 0.375, P = 0.045) (Figure 4). The mRNA expression of MCP-1 in urinary sediment correlated significantly with the degree of glomerulosclerosis (r = 0.450, P = 0.014) but not tubulointerstitial scarring (r = 0.316, P = 0.095) (Figure 4).

**Urinary cytokine level by ELISA**

Since the mRNA expressions of TGF-β and MCP-1 were significantly different between disease groups and controls, and correlated with histological damage, we went on to compare the urinary cytokine level by ELISA and the corresponding mRNA expression in urinary sediment. The TGF-β mRNA expression in urinary sediment did not correlate with its urinary level by ELISA (r = 0.024, P = 0.9), while there was a strong correlation between MCP-1 mRNA expression in urinary sediment and the corresponding urinary level by ELISA (r = 0.650, P < 0.001) (Figure 5).

Contrary to the mRNA expression in urinary sediment, neither the urinary TGF-β nor MCP-1 at the protein level correlated with renal function. However, urinary MCP-1 level by ELISA strongly correlated with the degree of proteinuria (r = 0.57, P = 0.001) and serum albumin level (r = −0.50, P = 0.006).

**Intra-renal cytokine expression**

Intra-renal expressions of TGF-β and MCP-1 were examined by immunohistochemistry. Detection of TGF-β and MCP-1 was found on the glomeruli and tubular and interstitial cells of the kidney biopsy specimens. The urinary mRNA expression of TGF-β significantly correlated with its glomerular (r = 0.701, P < 0.001) as well as tubulointerstitial TGF-β expression (r = 0.573, P = 0.001) as determined by immunohistochemistry (Figure 6). Urinary mRNA expression of MCP-1 had a modest correlation with its glomerular expression as determined by immunohistochemistry (r = 0.576, P = 0.001), but not the tubulointerstitial expression (r = −0.197, P = 0.306) (Figure 6).

**Urinary mRNA expression and renal function decline**

After being followed for 12 months, the urinary expressions of TGF-β and MCP-1 did not correlate...
with the rate of GFR decline. However, there was a significant inverse correlation between the rate of decline in estimated GFR and urinary expressions of CTGF ($r=-0.471$, $P=0.010$) and collagen I ($r=-0.399$, $P=0.032$) (Figure 7), but not with the expression of other target genes (Table 2).

**Discussion**

In this study, we found that the mRNA expressions of TGF-β, MCP-1 and collagen IV in urinary sediment were significantly different between diseased groups and controls. When only diseased groups were examined, urinary expression of TGF-β correlated significantly with baseline renal function and the degree of tubulointerstitial fibrosis, while MCP-1 expression correlated with the degree of glomerulosclerosis. The mRNA expression of MCP-1, but not TGF-β, correlated with its urinary level by ELISA. Urinary TGF-β mRNA correlated with both glomerular and
Fig. 5. Relationship between cytokine mRNA expression in urinary sediment and the corresponding urinary cytokine level by ELISA. (A) TGF-β and (B) MCP-1. Gene expressions are expressed as the ratio to the average expression of the control group.

Fig. 6. Relations between cytokine mRNA expression in urinary sediment and intra-renal expression by immunohistochemistry. (A) TGF-β and glomerular staining, (B) TGF-β and tubulointerstitial staining, (C) MCP-1 and glomerular staining and (D) MCP-1 and tubulointerstitial staining. Gene expressions are expressed as the ratio to the average expression of the control group.
tubulointerstitial expression by immunohistochemistry, while urinary MCP-1 mRNA correlated with glomerular expression only.

Although it was not the original objective of the present study, our data suggest the possibility of gene expression screening as a diagnostic test for specific glomerular diseases. For example, we found that the expressions of fibronectin, collagen III and HGF in urinary sediment were increased in patients with IgA nephropathy but not simple glomerulosclerosis, while the expressions of TGF-β and MCP-1 are increased in glomerulosclerosis but not IgA nephropathy (Figure 2). However, our present study examined only two diagnostic categories. The role of urinary gene expression study in the diagnosis of kidney disease requires validation by further large scale study involving a wide spectrum of disease entity.

Amongst the 11 target genes studied, we identified three (TGF-β, MCP-1 and collagen IV) whose expressions were significantly different between diseased groups and healthy controls and another three (CTGF, HGF and collagen III) with marginal difference. Although the number of patients studied was small, the magnitude of difference between the disease groups and healthy controls argued against a chance finding. Our observation is also consistent with published literature. TGF-β and MCP-1 play cardinal roles in the progression of renal failure [1,4–7] and the roles of CTGF and HGF are increasingly being recognized [16,17]. Although the Sclerosis Group had higher TGF-β and lower HGF mRNA expression in urinary sediment than the IgAN Group, the actual difference was only marginal and can be explained by the difference in the degree of glomerulosclerosis and tubulointerstitial scarring in the two groups (Table 1). Our data essentially show that the majority of the target gene mRNA expressions in urinary sediment examined in the present study were not disease specific. It is, however, important to note that we only studied patients with two types of chronic kidney diseases, namely IgA nephropathy and glomerulosclerosis. We did not study other types of glomerulonephritis, because they were less common causes of dialysis-dependent renal failure [18] and the presence of reversible active inflammation in other glomerulonephritis may affect the result [11]. Although diabetic nephropathy is a very common cause of dialysis-dependent renal failure, renal biopsy is generally not indicated for patients with typical presentation [19].

Amongst the potential candidate genes identified above, MCP-1 expression correlated with the degree of glomerulosclerosis, while TGF-β and CTGF correlated with tubulointerstitial fibrosis. It is interesting to note that TGF-β and CTGF expression did not associate with proteinuria, although it is accepted widely that proteinuria and renal scarring are closely related [1]. Our findings suggest that urinary TGF-β and CTGF mRNA expression and proteinuria are independent markers of renal damage.

We found that there was good internal correlation between urinary MCP-1 mRNA expression and its urinary level by ELISA, but the correlation between urinary TGF-β mRNA and the corresponding urinary level by ELISA was poor. There are several potential explanations. Firstly, urinary levels of TGF-β or MCP-1 detected by ELISA may not reflect local kidney production, because the test also detects leakage of TGF-β or MCP-1 from systemic circulation. The correlation between urinary MCP-1 levels by ELISA and the degree of proteinuria provides circumstantial evidence for this possibility. Secondly, renal tubular cells are organized into highly specialized luminal and basolateral compartments and the amount of cytokine protein found in urine may not correlate with the local concentration in renal interstitium [9]. ELISA may detect pre-formed cytokine and does not necessarily reflect immediate local production. This phenomenon
Urinary mRNA in CKD

is particularly relevant for TGF-β, since most of its tissue protein is synthesized at rest, binds to TGF latency-associated protein, but remains inactive [20]. Taken together, our data suggest that the measurement of cytokine gene expression in urinary sediment provides clinically relevant information in the assessment of histological damage, which cannot be substituted by the measurement of the corresponding urinary cytokine level by ELISA. We found that the rate of renal function decline correlated with the urinary expression of CTGF and collagen I, but not TGF-β or MCP-1. However, the follow-up period of our present study was short and it did not have adequate statistical power to confirm or exclude the prognostic role of any target gene. Unfortunately, we did not have follow-up data on the serial change in mRNA expression in our patients. Since the urinary TGF-β protein level reduces after angiotensin-receptor antagonist therapy in diabetic nephropathy [21] and chronic glomerulonephritis [22], it would be interesting to study the value of measuring cytokine mRNA expression in urinary sediment in the follow-up monitoring of patients with chronic kidney diseases.

In the present study, we could not affirm the cellular compositions of urinary sediment. However, urine microscopic examination was performed in all cases and urinary sediments were composed mainly of erythrocytes, mononuclear leukocytes and tubular epithelial cells. We believe the increased expression of most of the target genes came from mononuclear leukocytes as well as tubular epithelial cells.

In summary, amongst the target genes examined in the present study, the urinary expression of TGF-β had the best relation with renal function, degree of histological damage and intra-renal cytokine level. Measurement of TGF-β mRNA expression in urine may be a useful non-invasive tool for assessing the severity of renal damage in patients with chronic kidney diseases.

Acknowledgements. This study was supported in part by the CUHK research accounts 6901031 and 6900570.

Conflict of interest statement. None declared.

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Received for publication: 12.12.03
Accepted in revised form: 6.10.04