Urinary C-X-C motif chemokine 13 is a noninvasive biomarker of antibody-mediated renal allograft rejection

DAJIN CHEN*, JIAN ZHANG*, WENHAN PENG, CHUNHUA WENG and JIANGHUA CHEN

Kidney Disease Center, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003, P.R. China

Received November 22, 2017; Accepted April 23, 2018

DOI: 10.3892/mmr.2018.9211

Abstract. Noninvasive monitoring methods of immune status are preferred by transplant recipients. The present study investigated whether urinary C-X-C motif chemokine 13 (CXCL13) had the potential to reflect ongoing immune processes within renal allografts. Using an ELISA assay, the level of urinary CXCL13 was quantified in a total of 146 renal allograft recipients and 40 healthy controls at scheduled intervals and at the time of the indicated or protocol biopsy. The results of the present study revealed that urinary CXCL13/creatinine (Cr) was lower in normal transplants compared with in those with acute tubular necrosis (ATN; P=0.001), chronic allograft nephropathy (CAN; P=0.01), and acute rejection (AR; P<0.0001), which was associated with a good diagnostic performance for AR [area under the curve (AUC)=0.818, P<0.0001). In addition, urinary CXCL13/Cr levels in patients with AR were also higher than that of patients with graft dysfunction but no rejection, including ATN and CAN (P=0.034). Notably, urinary CXCL13 distinguished between acute antibody-mediated rejection (ABMR) and acute cellular rejection, with an AUC of 0.856. Furthermore, patients with steroid-resistant AR exhibited significantly increased urinary CXCL13/Cr levels than patients with reversible AR (P=0.001). Additionally, elevated levels of urinary CXCL13/Cr within the first month of transplant were predictive of graft function at 3 and 6 months (P=0.044 and P=0.04, respectively). Collectively, the findings of the present study indicated that the noninvasive investigation of urinary CXCL13/Cr may be valuable for the detection of AR, particularly ABMR. In addition, high urinary CXCL13/Cr levels predicted a poor response to steroid treatment and compromised graft function.

Correspondence to: Professor Jianghua Chen, Kidney Disease Center, The First Affiliated Hospital, College of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou, Zhejiang 310003, P.R. China
E-mail: chenjianghua@zju.edu.cn

*Contributed equally

Key words: C-X-C motif chemokine 13, kidney transplantation, noninvasive, rejection, urine

Introduction

Kidney transplantation has been established as the optimal renal replacement therapy for end-stage renal diseases, which surpasses dialysis treatment for the quality and quantity of life, as well as cost-effectiveness (1); however, rejection episodes and infection remain the major obstacles associated with kidney transplantation. Based on the increasing application of non-invasive monitoring technologies, the diagnosis of renal insufficiency during the early stages post-transplantation has progressed considerably in past decades; however, specific biomarkers for the noninvasive diagnosis/prognosis of acute rejection (AR), particularly antibody-mediated rejection (ABMR), are yet to be identified.

Chemokine (C-X-C motif) ligand 13 (CXCL13), otherwise known as B cell-attracting chemokine 1, is a small cytokine belonging to the CXC chemokine superfamily (2). As its name suggests, CXCL13 is selectively chemotactic for circulating B cells by interacting with chemokine receptor (CXCR)5 (3,4), a 7-transmembrane G-protein-coupled receptor expressed on the surface of mature B cells and a subset of memory cells (5,6). The gene for CXCL13 is located on human chromosome 4 in a cluster with other CXC chemokines (7), including interleukin-8 and interferon-inducible protein 10.

Recent studies suggest that the ligation of CXCR5 with CXCL13 may lead to an aberrant aggregation of B cells, which has been confirmed in rheumatoid arthritis, gastric lymphoma, and central nervous system lymphoma (8-11). Additionally, a notable colocalization of CXCL13 expression with CXCR5 and cluster of differentiation 20-positive B lymphocytes has also been detected in renal allografts undergoing rejection (12,13). In addition, our previous study revealed that CXCL13 expression levels are highly upregulated in the peripheral blood mononuclear cells of patients with AR (14).

The particular interaction between CXCL13 and B lymphocytes, as well as the significance of B cell infiltration in transplant rejection, may be detected noninvasively as an intragraft signature by measuring the levels of CXCL13 in urine samples. Furthermore, such an investigation may contribute to the monitoring of immune kinetics. To test this hypothesis, the urinary protein expression levels of CXCL13 were measured following renal transplantation in a total of 146 renal allograft recipients. The present study examined whether urinary CXCL13 may effectively identify AR, in particular ABMR. Secondly, whether

the early elevation of urinary CXCL13 expression levels may serve as an indication of graft function was investigated.

Materials and methods

Study population and sample collection. The present study retrospectively reviewed 146 patients (aged 22-69 years old; 97 males and 49 females) who received a single kidney transplantation from donors who had succumbed to mortality between June 2006 and December 2009 at the Kidney Disease Center, the First Affiliated Hospital of College of Medicine of Zhejiang University (Hangzhou, China). Fresh first-morning urine specimens were routinely collected from the patients every 2 weeks during the first 2 months following transplantation. In addition, urine samples were collected from patients who had undergone transplantation and were scheduled for a biopsy with a serum creatinine (Scr) concentration ≥25% above baseline levels post-transplantation, and from patients who were to undergo a protocol needle biopsy with stable renal function within 2-3 months post-transplantation. Furthermore, urine samples were obtained from 36 patients with stable renal function and 21 patients with AR every day within the first week and at a one-week interval up to the first month post-transplantation. According to the mean urinary CXCL13/Cr levels (>2 pg/μmol Cr or <2 pg/μmol Cr) exhibited by patients within the first week post-transplantation, patients were separated into two groups: High CXCL13/Cr levels group (>2 pg/μmol Cr) and low CXCL13/Cr levels (<2 pg/μmol Cr) group. For these 57 patients, renal function at 3, 6 and 12 month time intervals post-transplantation were investigated. On the day of the biopsy, urinary samples were collected prior to biopsy collection. Furthermore, 40 healthy individuals were included as controls (aged 27-65 years old; 24 males and 16 females) that did not exhibit any signs of infection or malignant tumors. On the first day of recruitment, one urine sample was collected from each patient. All patients were assigned to a diagnostic category based on an aggregate of all available diagnostic data, including clinical and pathological manifestation as determined by histological analysis (15).

Patients classified in the AR group exhibited histological alterations following H&E and Periodic Acid-Schiff (PAS) staining. Immunohistochemical analysis was performed using the Cd4 stain. For H&E and PAS staining, renal tissues were fixed in a 4% formaldehyde solution for 48 h at 24˚C and then embedded in paraffin. Sections (4 μm) were both stained using H&E stain at 24˚C for 2 h and PAS stain at 24˚C for 3 h. For IHC staining, paraffin embedded sections (4 μm), fixed in a 4% formaldehyde solution for 48 h at 24˚C, and subsequently incubated for 30 min at 37˚C with a polyclonal rabbit anti-human C4d antibody (cat. no. BI-RC4D; 1:50; Biomedica, Inc., Vienna, Austria). Following this, sections were blocked with 5% bovine serum albumin (cat. no. A1933; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 24˚C for 30 min. Following three washes with PBS, sections were incubated for 30 min at 24˚C with peroxidase AffiniPure goat anti-rabbit secondary antibodies (cat. no. 111-035-003; 1:4,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Antibody detection was performed using a DAB Horseradish Peroxidase Color Development Kit (cat. no. GA042329; Gene Tech Co., Ltd., Hong Kong, China). Images of these stained sections were obtained using a light microscope (magnification, x200 and x400). Classification was performed using the Banff 97 criteria (16), while meeting the clinical criteria associated with renal dysfunction (Scr elevation of ≥25% above baseline within 6 months post-transplant); patients classified as stable renal transplant manifested as normal allograft function and no abnormal pathological findings (NO-AR) in the protocol biopsies performed 2-3 months following transplantation. Primary grafts were also received from deceased donors.

All patients provided written informed consent. All procedures performed in studies involving human participants were approved by the Ethics Committee of the First Affiliated Hospital of College of Medicine of Zhejiang University (Hangzhou, China) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (Reference number: 2017-390).

In the present study, immunosuppressive agents were used as previously described (17,18). All patients received a regimen of three immunosuppressive drugs at the time of transplantation, comprising a calcineurin inhibitor (tacrolimus), prednisone and azathioprine or mycophenolate mofetil. Anti-rejection therapy following a clinical and biopsy-proven diagnosis of AR constituted a 3-day course of intravenous methylprednisolone (6-10 mg/kg per day, once a day). A lack of response to steroid treatment (graft function exhibited no improvement or worsened) was defined as steroid-resistant AR (SRAR). Histology was classified according to the Banff 97 classification (16,19) and was performed by two experienced renal pathologists in a blinded fashion.

Fresh urinary samples were collected and centrifuged for 10 min at 800 x g at 4˚C, using a D-37520 Sorvall Legend RT centrifuge (Heraeus Holding GmbH, Hanau, Germany). The supernatant was frozen in 1 ml aliquots at -80˚C. Urinary Cr and protein were detected in all samples.

ELISA: Quantification of CXCL13 in urine samples. The expression levels of CXCL13 were measured in urinary samples using a commercial human CXCL13 ELISA kit, according to the manufacturer's protocols (R&D Systems, Inc., Minneapolis, MN, USA, cat. no. DCX130). All samples were undiluted and analyzed in duplicate.

Statistical analysis. To eliminate the influence of renal function on urinary protein quantitation, all urinary CXCL13 levels were normalized to urine creatinine (Cr) in the present study. Summary statistics for normally distributed quantitative variables were expressed as the mean ± standard deviation. For non-normally distributed variables, we used the median and interquartile range (IQR). Differences in the continuous variables were judged using a Mann-Whitney U test or Kruskal-Wallis H test followed by Tukey's post hoc test. A conventional receiver operating characteristic (ROC) curve was conducted to determine the sensitivities and specificities for patients with and without AR. Youden's index, defined as sensitivity + specificity-1, was used to calculate the diagnostic threshold. All statistical analyses were performed using SPSS software package (version 23.0; IBM Corp., Armonk, NY, USA), and a two-sided $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Baseline characteristics of transplant recipients, grouped according to histological results.

Variables	AR (n=49)	ATN (n=10)	CAN (n=29)	NO-AR (n=58)
Mean age (mean ± standard deviation)	36.9±9.6	37.3±5.8	45.8±9.5	39.8±10.1
Age range	25-59	27-49	23-69	22-57
Gender, n (%)				
Male	34 (69.4%)	7 (70.0%)	20 (68.9%)	36 (62.1%)
Female	15 (30.6%)	3 (30.0%)	9 (31.1%)	22 (37.9%)
Cause of ESRD, n (%)				
Glomerulonephritis	38 (77.6%)	8 (80.0%)	24 (82.7%)	44 (75.9%)
Hypertension	2 (4.1%)	0	1 (3.4%)	2 (3.4%)
Obstructive uropathy	1 (2.1%)	0	0	1 (1.7%)
Diabetes	4 (8.1%)	1 (10.0%)	2 (6.9%)	4 (6.8%)
Others	4 (8.1%)	1 (10.0%)	2 (7.0%)	7 (12.1%)
Dialysis time (mean ± standard deviation)	6.5±4.1	5.3±2.8	6.3±4.7	6.9±7.1
HLA mismatch (mean ± standard deviation)	3.6±1.3	3.5±1.2	3.9±1.5	3.2±1.3
Cold ischemia (mean ± standard deviation)	8.3±1.9	8.6±2.0	8.5±2.1	8.1±1.6
Panel reactive antibody, n (%)				
<10%	44 (89.8%)	9 (90.0%)	26 (89.7%)	(93.1%)
>10%	5 (10.2%)	1 (10.0%)	3 (10.3%)	4 (6.9%)

AR, acute rejection; ATN, acute tubular necrosis; CAN, chronic allograft nephropathy; ESRD, end-stage renal disease; HLA, human leucocyte antigen; NO-AR, normal allograft function and no abnormal pathological findings; SCR, subclinical rejection.

Results

Patients and baseline clinical and biopsy characteristics. A total of 146 patients who had both biopsy results and matched urine samples were analyzed in the present study, including 49 patients with biopsy-proved AR. Among the 49 patients with AR, 37 were diagnosed as acute cellular rejection (ACR), exhibiting significant infiltration of interstitial mononuclear cells, including >25% of parenchyma affected and moderate (>4 mononuclear cells/tubular cross section) to severe tubulitis (>10 mononuclear cells/tubular cross section); and 12 were diagnosed with ABMR, exhibiting microvascular inflammation or arteritis and positive C4d staining results (data not shown). Demographic information for the four histologically-defined groups is summarized in Table I, and no differences in the baseline characteristics were observed (P>0.05). The remaining 97 biopsy specimens revealed NO-AR patients (n=58) in the protocol biopsy, and dysfunction with no rejection (DNR, n=39), including acute tubular necrosis (ATN; n=10) and chronic allograft nephropathy (CAN; n=29) in the indication biopsy.

Urinary CXCL13/Cr is a diagnostic biomarker of acute renal allograft rejection. As presented in Fig. 1, the CXCL13/Cr urinary levels were significantly different between the diagnostic groups (P<0.0001). Urinary CXCL13/Cr was notably low in NO-AR (median: 0.244; IQR, 0.164–0.562 pg/μmol Cr) and healthy control subjects (median: 0.275; IQR, 0.165-0.409 pg/μmol Cr) (P=0.772). Conversely, the levels of CXCL13/Cr in the urine were differentially elevated in patients with ATN (median: 2.46; IQR, 1.370-3.775 pg/μmol Cr; P<0.001), CAN (median: 0.706; IQR, 0.278-2.309 pg/μmol Cr) (P=0.01), and AR in particular (median: 2.438; IQR, 0.802-8.261 pg/μmol Cr); P<0.0001) compared with patients with NO-AR. It is important to note that the urinary CXCL13/Cr levels in patients with AR were significantly higher than that of patients with DNR (ATN + CAN; P=0.034).

A ROC curve analysis for CXCL13/Cr was conducted to assess its performance in the diagnosis of AR (Fig. 2), compared with the diagnosis of NO-AR. The results revealed that CXCL13/Cr yielded a good diagnostic power, with an area under the curve (AUC) of 0.818 [95% confidence interval (CI): 0.732-0.903]. At the cut point for optimizing the diagnostic effect, the sensitivity and the specificity reached 84 and 79%, respectively. In addition, the analysis was repeated while distinguishing AR from the DNR diagnosis, which yielded an AUC of 0.632 (95% CI: 0.516-0.748; P=0.034).

Urinary CXCL13/Cr level identifies ABMR. Among the 49 patients with AR, 37 were diagnosed as ACR and 12 were ABMR according to the antibody-mediated rejection criteria. As expected, the levels of urinary CXCL13/Cr in the patients with ABMR (median: 18.559; IQR: 5.206-35.281 pg/μmol Cr) were significantly higher than those with ACR (median: 1.237; IQR: 0.686-4.425 pg/μmol Cr) (Fig. 3).

A ROC curve was performed to determine the discriminatory capacity of CXCL13/Cr levels for ABMR. The results indicated that urinary CXCL13/Cr effectively distinguished ABMR from ACR with an AUC of 0.856 (95% CI: 0.701-1.0; Fig. 4).

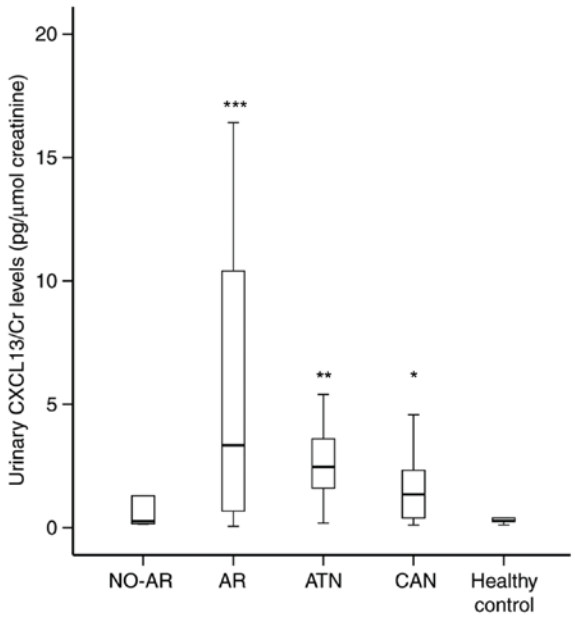

Figure 1. Median concentration of urinary CXCL13/Cr in patients with AR, ATN, CAN, NO-AR, and healthy controls. Among the five groups, there was a significant statistical difference in the CXCL13/Cr levels in the urine. The graph shows that urinary CXCL13/Cr levels were significantly elevated in patients with CAN (n=29), ATN (n=10), and AR (n=49), compared with in NO-AR patients (n=58), *P<0.01, **P<0.001, and ***P<0.0001, respectively. ATN, acute tubular necrosis; AR, acute rejection; CAN, chronic allograft nephropathy; Cr, creatinine; CXCL13, C-X-C motif chemokine 13; NO-AR, patients with stable renal function.

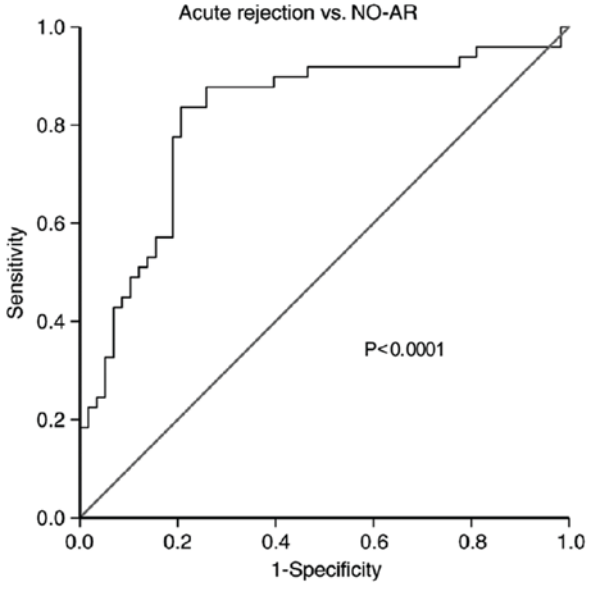

Figure 2. Analysis by a ROC curve for urinary CXCL13/Cr as a marker of diagnosis of acute rejection. The area under ROC curve was 0.818 (95% confidence interval: 0.732-0.903; P<0.0001). Cr, creatinine; CXCL13, C-X-C motif chemokine 13; ROC, receiver operating characteristic.

Levels of urinary CXCL13/Cr are associated with the response to anti-rejection treatment and prognosis. The present study analyzed the association between CXCL13/Cr levels in the urine specimens obtained prior to biopsy with the response to anti-rejection therapy. As aforementioned, the AR recipients

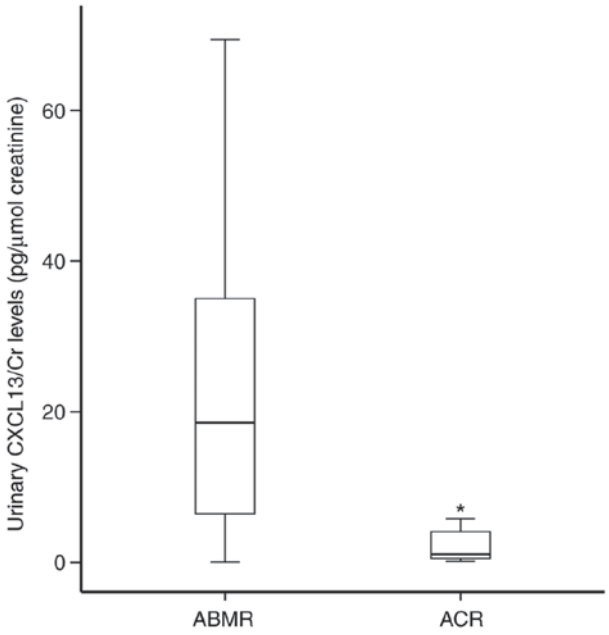

Figure 3. Median concentration of urinary CXCL13/Cr in renal transplant patients with ABMR and ACR. Patients with ABMR excreted urinary CXCL13/Cr at a significantly higher level than those with ACR. *P<0.0001 vs. ACR. ABMR, antibody mediated rejection; ACR, acute cellular rejection; Cr, creatinine; CXCL13, C-X-C motif chemokine 13.

received anti-rejection treatment with high-dose methylprednisolone. There were 18 patients who demonstrated a positive response to anti-rejection treatment, and Cr declined to baseline levels following anti-rejection therapy (steroid-sensitive). Conversely, 31 recipients showed a poor response to anti-rejection treatment, in whom Cr did not recover to baseline levels and were maintained above 200 μmol/l following anti-rejection therapy (steroid-resistant). Additionally, 6 patients lost renal function and returned to dialysis after 3 months. Compared with patients with steroid-sensitive AR, urinary CXCL13/Cr levels were significantly higher in patients with SRAR (median: 5.55; IQR: 1.508-11.874; P<0.001), as presented in Fig. 5. Therefore, urinary CXCL13/Cr may also be used to detect steroid-resistant rejection among patients with AR. An ROC analysis was performed, which revealed that the CXCL13/Cr levels in the urine may be a moderately good predictor of a poor response to anti-rejection treatment, with an AUC of 0.810 (95% CI: 0.676-0.945). Based on the ROC curve (Fig. 5), a CXCL13/Cr value of 0.9 was associated with 90% sensitivity and 67% specificity for detecting a patient with steroid-resistant rejection; however, no significant discrepancy was observed between the recipients with allograft dysfunction and reversible acute rejection (P=0.259).

Post-transplantation urinary CXCL13/Cr levels are associated with restricted graft outcome. Urine samples were obtained from 36 patients with stable renal function and 21 patients with AR every day within the first week and at a one-week interval up to the first month post-transplantation. These samples were additionally used to investigate whether elevated CXCL13/Cr levels in the urine within the first month post-transplantation were predictive of graft function after 3, 6 and 12 months. The 57 patients were divided into two groups according to

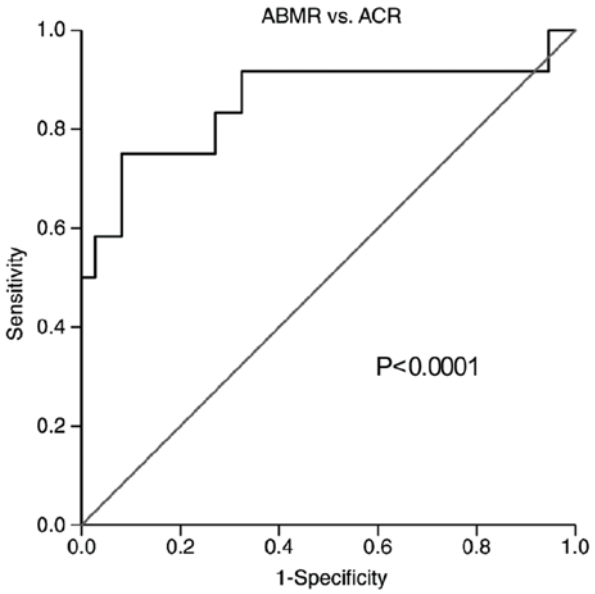

Figure 4. Analysis by a ROC curve for urinary CXCL13/Cr as a marker of a diagnosis of ABMR. The area under ROC curve was 0.856 (95% confidence interval: 0.701-1.0; P<0.0001), which revealed that urinary CXCL13/Cr was a suitable marker for the diagnosis of ABMR. ABMR, antibody mediated rejection; ACR, acute cellular rejection; ROC receiver operating characteristic.

the individual mean urinary CXCL13/Cr levels within the first month post-transplantation; 2 pg/μmol Cr was defined as the cutoff value. With an exception of 12 months, the 3 and 6-month estimated glomerular filtration rates were significantly lower in patients with high CXCL13/Cr levels (>2 pg/μmol Cr) compared with in patients with low CXCL13/Cr levels (<2 pg/μmol Cr; Fig. 6). In addition, whether elevated levels of urinary CXCL13/Cr during the first month post-transplantation were associated with poor graft function independent of AR was investigated in the present study. Urinary CXCL13/Cr levels of the patients in the group without any signs of clinical rejection were analyzed separately. The results of the analysis indicated that there was no significant difference in the graft function between patients with high urinary CXCL13/Cr and the other patients throughout the entire first year (data not shown).

Discussion

The accurate and timely detection of transplant rejection and effective therapy are essential for the long-term survival of kidney transplant patients. Measurement of Scr following kidney transplantation is one of the most widely used methods of monitoring renal allograft function; however, the elevation of Scr is a relatively late event of intragraft injury (20).

Some publications have revealed that distinct alterations occur in the levels of certain proteins in the urine obtained from transplant patients during AR, which are notably pronounced compared with alterations in Scr. For example, Hu *et al* (21) reported that CXCL9 and CXCL10 are associated with acute renal injury, by screening urine samples for 23 types of chemokines and cytokines. Matz *et al* (20) further demonstrated that CXCL-10 expression in the urine may be

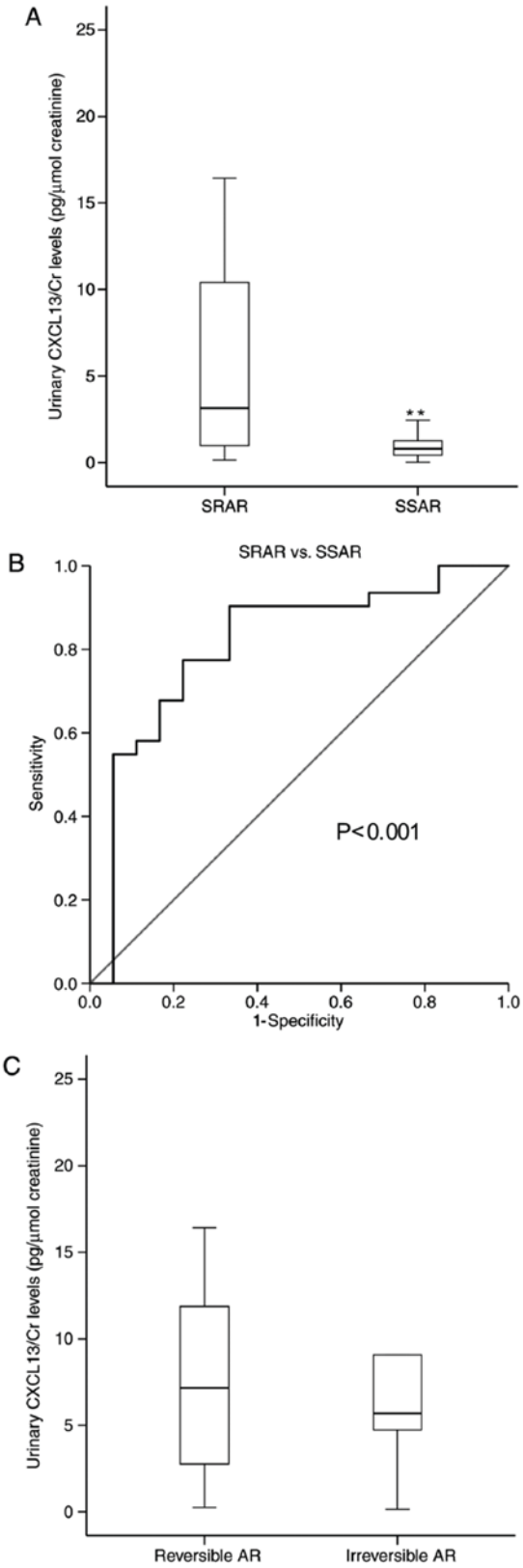

Figure 5. Urinary CXCL13/Cr levels were differentially expressed between patients with SRAR and SSAR. (A) Urinary CXCL13/Cr levels in patients with SRAR (n=31) were higher than those with SSAR (n=18) **P<0.001. (B) ROC curve for urinary CXCL13/Cr as a marker for distinguishing SRAR from SSAR. The area under the ROC curve was 0.810 (95% confidence interval: 0.676-0.945; P<0.001). (C) Urinary CXCL13/Cr levels were not significantly different between the recipients with an allograft dysfunction and those with reversible AR. AR, acute rejection; Cr, creatinine; CXCL13, C-X-C motif chemokine 13; SSAR, steroid sensitivity acute rejection; SRAR, steroid resistant acute rejection.

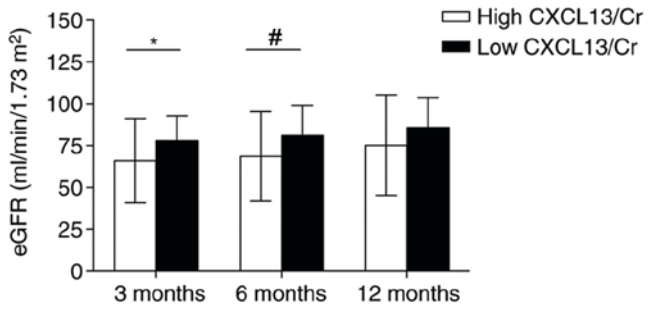

Figure 6. Compared with patients exhibiting low CXCL13/Cr levels (<2 pg/μmol Cr), patients exhibited greater mean CXCL13/Cr levels (>2 pg/μmol Cr) during the first month post-transplantation demonstrated significantly impaired 3- and 6-month graft function (*P=0.044 and $^{\#}$P<0.04, respectively) but not 12-month graft function (P>0.05). Cr, creatinine; CXCL13, C-X-C motif chemokine 13; eGFR, estimated glomerular filtration rate.

used to identify patients with ongoing AR episodes several days prior to a biopsy based on increasing Scr levels. In addition, data from a multicenter observational Clinical Trials in Organ Transplantation Protocol-01 (CTOT-01) (22) revealed that CXCL9 protein levels possess a strong predictive value for noninvasively diagnosing T cell-mediated rejection (TCMR). In addition, Ho *et al* (23) recently reported that elevated urinary matrix metalloproteinase-7 may be used to detect underlying renal allograft inflammation and injury, which may improve the overall diagnostic performance of urinary CXCL10 for distinguishing normal histology from subclinical and clinical injury.

ABMR has gradually become a major problem and is now considered to be the main cause of long-term allograft dysfunction compared with TCMR (24); however, few studies have succeeded in identifying specific biomarkers for the diagnosis and noninvasive serial monitoring of ABMR. Thus, ABMR is often underdiagnosed in clinical research and routine clinical practice. It was reported in the CTOT-01 study (22) that only two ABMRs and four mixed rejections were diagnosed among 150 indication biopsies.

Using quantitative polymerase chain reaction, we previously reported that CXCL13 expression levels are significantly elevated in the peripheral blood mononuclear cells of transplant rejection patients, which are also associated with a poor response to anti-rejection therapy (14). Furthermore, an expression analysis of biopsy specimens indicated that the intrarenal CXCL13 mRNA expression levels were 27-fold higher in transplants with B-cell clusters, compared with in rejecting allografts without B-cell aggregation. Collectively, these results suggest a potential role for CXCL13 in AR, particularly in ABMR (13).

The present study reported that transplant patients with AR episodes exhibited significantly increased urinary CXCL13 protein expression compared with patients with stable renal function, ATN, as well as CAN. It is important to note that the CXCL13 expression levels of almost all healthy controls were near the lowest threshold of detection. In addition, among patients with AR, the severity was associated with the CXCL13 levels in the urine, which may be explained by the existence of a positive feedback mechanism between CXCL13 and its receptor, CXCR5 (25). Additionally, higher CXCL13 levels in the urine of patients with ABMR were observed, compared with in patients with ACR. Therefore, the present study conducted an ROC curve to test the discriminatory power of CXCL13 for ABMR. The results revealed that AUC reached up to 0.856, with a cut point of 8.26 pg/μmol creatinine. These findings were consistent with the results of Steinmetz *et al* (13) and our previous study (14), which generated a convincing conclusion that the detection of urinary CXCL13 levels may provide a good basis for the clinical diagnosis of AR and more importantly, distinguish it from other patterns of rejection.

The prognostic attributes of CXCL13 expression for AR, particularly ABMR, were further analyzed by conducting kinetic observations of CXCL13 protein levels in urinary specimens obtained from a subset of transplant patients. The present study revealed that the 3 and 6-month renal function of patients with high levels of CXCL13/Cr post-transplantation was decreased compared with patients exhibiting low CXCL13/Cr levels; however, no variations were observed when analysis was limited to patients without AR. These data revealed an association between enhanced CXCL13 expression in the urine during the first month and intragraft immune activation, which may result in AR and subsequent compromised graft function.

The possible origin of CXCL13 has been investigated in previous studies. In murine secondary lymphoid organs, CXCL13 is primarily produced by stromal cells resident in B cell follicles, comprising follicular dendritic cells and marginal reticular cells (26,27). Conversely, germinal-center follicular helper T (Tfh) cells may be potent producers of CXCL13 in humans (28,29). The early induction of CXCL13 in the graft may be the result of tissue injury and innate immune activation leads to the infiltration of inflammatory cells (such as Tfh cells), which initiates the secretion of various chemokines, including CXCL13. The release of CXCL13 may further increase the recruitment of activated leukocytes to the graft in a self-sustaining positive feedback loop thereafter.

The primary strength of this assay is its non-invasiveness, which provides the opportunity for frequent, serial immune monitoring. Compared with blood biomarkers, urine markers have several advantages, including the non-invasive nature of sample collection and few interfering proteins. Thus, this assay can be performed in clinical practice to instruct clinical decision-making with respect to the requirement for a biopsy (separating patients into those that should undergo an immediate biopsy from those that may be followed-up and even taper their immunosuppressive therapy). Furthermore, this test may be used to predict patient responses to anti-rejection therapy.

The present study was associated with several limitations: Firstly, the clinical-pathological classification of all patients relied on an allograft histological examination, which may have been subject to sampling error. Secondly, this was a retrospective analysis and there was a lack of data on peritubular capillaritis, and donor-specific antibody status, to regroup all patients according to the updated Banff criteria (30). Thus, it is possible that mixed rejection phenotypes may have contaminated the ACR groups; however, the relatively small in the quantity of ABMR patients suggests that if the ACR group was contaminated with mixed rejection, the differences

in urinary CXCL13 levels between ACR and ABMR patients may have been underestimated in the present study. Finally, the cohort size of the present study was small, and these results will require validation in larger prospective cohorts.

In conclusion, in addition to confirming the feasibility of using urinary CXCL13 protein levels for the noninvasive diagnosis of AR, the results of the present study demonstrated that urinary CXCL13/Cr levels were highly associated with ABMR, which may serve to distinguish ABMR from ACR. Furthermore, patients with heightened urinary CXCL13/Cr were associated with a poor response to steroid treatment and restricted short-term graft function. Finally, the present study suggested that monitoring the expression of CXCL13 protein in the urine of renal transplant recipients may have contributed to early individualized rectifications of immunosuppressive therapy and therefore, lowered the incidence of severe graft damage.

Acknowledgements

The authors would like to thank the pathologists from the Kidney Disease Immunology Laboratory, the First Affiliated Hospital of Zhejiang University (Hangzhou, China).

Funding

The present study was supported by grants from the Medical and Health Technology Development Program in Zhejiang (grant no. 2014KYA057) and the Foundation of Zhejiang Provincial Natural Science Foundation of China (grant no. LQ16H050003).

Availability of data and materials

The original datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contribution

JC and DC designed the study. CW and WP performed sample collection. ZJ and DC performed sample collection, and acquired and analyzed the data. DC and ZJ contributed to the writing and editing of the manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were approved by the Ethics Committee of the First Affiliated Hospital of College of Medicine of Zhejiang University (Hangzhou, China). All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Garcia GG, Harden P and Chapman J; World Kidney Day Steering Committee 2012: The global role of kidney transplantation. Lancet 379: e36-e38, 2012.
2. Ezzat M, El-Gammasy T, Shaheen K and Shokr E: Elevated production of serum B-cell-attracting chemokine-1 (BCA-1/CXCL13) is correlated with childhood-onset lupus disease activity, severity, and renal involvement. Lupus 20: 845-854, 2011.
3. Hu H, Aizenstein BD, Puchalski A, Burmania JA, Hamawy MM and Knechtle SJ: Elevation of CXCR3-binding chemokines in urine indicates acute renal-allograft dysfunction. Am J Transplant 4: 432-437, 2004.
4. Velaga S, Herbrand H, Friedrichsen M, Jiong T, Dorsch M, Hoffmann MW, Förster R and Pabst O: Chemokine receptor CXCR5 supports solitary intestinal lymphoid tissue formation, B cell homing, and induction of intestinal IgA responses. J Immunol 182: 2610-2619, 2009.
5. Förster R, Mattis AE, Kremmer E, Wolf E, Brem G and Lipp M: A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. Cell 87: 1037-1047, 1996.
6. Förster R, Emrich T, Kremmer E and Lipp M: Expression of the G-protein-coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. Blood 84: 830-840, 1994.
7. Gunn MD, Ngo VN, Ansel KM, Ekland EH, Cyster JG and Williams LT: A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. Nature 391: 799-803, 1998.
8. Smith JR, Braziel RM, Paoletti S, Lipp M, Uguccioni M and Rosenbaum JT: Expression of B-cell-attracting chemokine 1 (CXCL13) by malignant lymphocytes and vascular endothelium in primary central nervous system lymphoma. Blood 101: 815-821, 2003.
9. Amft N, Curnow SJ, Scheel-Toellner D, Devadas A, Oates J, Crocker J, Hamburger J, Ainsworth J, Mathews J, Salmon M, *et al*: Ectopic expression of the B cell-attracting chemokine BCA-1 (CXCL13) on endothelial cells and within lymphoid follicles contributes to the establishment of germinal center-like structures in Sjogren's syndrome. Arthritis Rheum 44: 2633-2641, 2001.
10. Shi K, Hayashida K, Kaneko M, Hashimoto J, Tomita T, Lipsky PE, Yoshikawa H and Ochi T: Lymphoid chemokine B cell-attracting chemokine-1 (CXCL13) is expressed in germinal center of ectopic lymphoid follicles within the synovium of chronic arthritis patients. J Immunol 166: 650-655, 2001.
11. Mazzucchelli L, Blaser A, Kappeler A, Schärli P, Laissue JA, Baggiolini M and Uguccioni M: BCA-1 is highly expressed in Helicobacter pylori-induced mucosa-associated lymphoid tissue and gastric lymphoma. J Clin Invest 104: R49-R54, 1999.
12. Steinmetz OM, Stahl RA and Panzer U: Chemokines and B cells in renal inflammation and allograft rejection. Front Biosci (Schol Ed) 1: 13-22, 2009.
13. Steinmetz OM, Panzer U, Kneissler U, Harendza S, Lipp M, Helmchen U and Stahl RA: BCA-1/CXCL13 expression is associated with CXCR5-positive B-cell cluster formation in acute renal transplant rejection. Kidney Int 67: 1616-1621, 2005.
14. Mao Y, Wang M, Zhou Q, Jin J, Wang Y, Peng W, Wu J, Shou Z and Chen J: CXCL10 and CXCL13 Expression were highly up-regulated in peripheral blood mononuclear cells in acute rejection and poor response to anti-rejection therapy. J Clin Immunol 31: 414-418, 2011.
15. Chen D, Peng W, Jiang H, Yang H, Wu J, Wang H and Chen J: Noninvasive detection of acute renal allograft rejection by measurement of soluble Tim-3 in urine. Mol Med Rep 16: 915-921, 2017.
16. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, *et al*: The Banff 97 working classification of renal allograft pathology. Kidney Int 55: 713-723, 1999.
17. Peng W, Chen J, Jiang Y, Shou Z, Chen Y and Wang H: Acute renal allograft rejection is associated with increased levels of vascular endothelial growth factor in the urine. Nephrology (Carlton, Vic.) 13: 73-79, 2008.
18. Wu JY, Chen JH, Wang YM, He Q and Wu DB: Improved clinical outcomes in Chinese renal allograft recipients receiving lower dose immunosuppressants. Transplantation 78: 713-718, 2004.
19. Racusen LC, Halloran PF and Solez K: Banff 2003 meeting report: New diagnostic insights and standards. Am J Transplant 4: 1562-1566, 2004.

20. Matz M, Beyer J, Wunsch D, Mashreghi MF, Seiler M, Pratschke J, Babel N, Volk HD, Reinke P and Kotsch K: Early post-transplant urinary IP-10 expression after kidney transplantation is predictive of short- and long-term graft function. Kidney Int 69: 1683-1690, 2006.
21. Hu H, Kwun J, Aizenstein BD and Knechtle SJ: Noninvasive detection of acute and chronic injuries in human renal transplant by elevation of multiple cytokines/chemokines in urine. Transplantation 87: 1814-1820, 2009.
22. Hricik DE, Nickerson P, Formica RN, Poggio ED, Rush D, Newell KA, Goebel J, Gibson IW, Fairchild RL, Riggs M, *et al*: Multicenter validation of urinary CXCL9 as a risk-stratifying biomarker for kidney transplant injury. Am J Transplant 13: 2634-2644, 2013.
23. Ho J, Rush DN, Krokhin O, *et al*: Elevated urinary matrix metalloproteinase-7 detects underlying renal allograft inflammation and injury. Transplantation 100: 648-654, 2016.
24. Rabant M, Amrouche L, Lebreton X, Aulagnon F, Benon A, Sauvaget V, Bonifay R, Morin L, Scemla A, Delville M, *et al*: Urinary C-X-C motif chemokine 10 independently improves the noninvasive diagnosis of antibody-mediated kidney allograft rejection. J Am Soc Nephrol 26: 2840-2851, 2015.
25. Jenh CH, Cox MA, Hipkin W, Lu T, Pugliese-Sivo C, Gonsiorek W, Chou CC, Narula SK and Zavodny PJ: Human B cell-attracting chemokine 1 (BCA-1; CXCL13) is an agonist for the human CXCR3 receptor. Cytokine 15: 113-121, 2001.
26. Kielczewski JL, Horai R, Jittayasothorn Y, Chan CC and Caspi RR: Tertiary lymphoid tissue forms in retinas of mice with spontaneous autoimmune uveitis and has consequences on visual function. J Immunol 196: 1013-1025, 2016.
27. Crotty S: Follicular helper CD4 T cells (TFH). Ann Rev Immunol 29: 621-663, 2011.
28. Kim CH, Lim HW, Kim JR, Rott L, Hillsamer P and Butcher EC: Unique gene expression program of human germinal center T helper cells. Blood 104: 1952-1960, 2004.
29. Rasheed AU, Rahn HP, Sallusto F, Lipp M and Muller G: Follicular B helper T cell activity is confined to CXCR5(hi) ICOS(hi) CD4 T cells and is independent of CD57 expression. Eur J Immunol 36: 1892-1903, 2006.
30. Haas M, Sis B, Racusen LC, Solez K, Glotz D, Colvin RB, Castro MC, David DS, David-Neto E, Bagnasco SM, *et al*: Banff 2013 meeting report: Inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. Am J Transpl 14: 272-283, 2014.