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MINIREVIEWS

Role for urinary biomarkers in diagnosis of acute rejection in the transplanted kidney

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Abstract

Despite the introduction of potent immunosuppressive medications within recent decades, acute rejection still accounts for up to 12% of all graft losses, and is generally associated with an increased risk of late graft failure. Current detection of acute rejection relies on frequent monitoring of the serum creatinine followed by a diagnostic renal biopsy. This strategy is flawed since an alteration in the serum creatinine is a late clinical event and significant irreversible histologic damage has often already occurred. Furthermore, biopsies are invasive procedures that carry their own inherent risk. The discovery of non-invasive urinary biomarkers to help diagnose acute rejection has been the subject of a significant amount of investigation. We review the literature on urinary biomarkers here, focusing on specific markers perforin and granzyme B mRNAs, FOXP3 mRNA, CXCL9/CXCL10 and miRNAs. These and other biomarkers are not yet widely used in clinical settings, but our review of the literature suggests that biomarkers may correlate with biopsy findings and provide an important early indicator of rejection, allowing more rapid treatment and better graft survival.

Key words: Urinary biomarkers; Acute renal allograft rejection; Serum creatinine; Graft outcome; Urinary perforin, granzyme B and Fas-ligand mRNA; Urinary CXCL9 and CXCL10; Urinary FOXP3 mRNA; Urinary miRNA

Core tip: Through its urine output, the transplanted kidney can provide a window into the cellular and molecular events occurring within the graft, and potentially offers a noninvasive means of assessing

kidney allograft status. An assay consisting of biomarkers of allograft injury using only urine samples from transplant recipients could provide many advantages over the current strategy of relying on changes in the serum creatinine and kidney biopsies. A rising creatinine is a nonspecific marker of graft dysfunction and a relative late marker of intragraft pathology, whereas kidney biopsies are inherently invasive. The role of non-invasive monitoring through plasma or urine biomarkers has been a topic of interest to the transplant community for many years and has been the subject of numerous publications. Our objective is to critically review the current literature to better delineate the role of these urinary biomarkers in predicting the risk of acute allograft rejection in kidney transplant recipients.

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INTRODUCTION

For many people whose renal disease has progressed to end stage, kidney transplantation offers a greater survival advantage and better quality of life compared to hemo or peritoneal dialysis[1]. Even with the introduction of improved immunosuppressive drugs and regimens in recent decades, acute cellular or antibody-mediate rejection remains a persistent threat to allograft survival. Some 12% of all graft loss is due to acute rejection (AR), particularly in the first six months after transplantation[2]. Even with prompt therapy, AR is associated with reduced allograft survival[3]. In a review of 48179 kidney transplant recipients between 2000 and 2007, AR within the first year of transplantation carried more than a five-fold adjusted relative risk for all-cause graft loss compared to unaffected individuals[4]. AR is also a major risk factor for chronic allograft nephropathy, defined histologically by interstitial fibrosis and tubular atrophy (IFTA). AR is the primary cause of graft loss beyond the first year[5].

AR represents an acute functional decline in the transplanted kidney associated with specific histopathologic changes resulting from an active immune response on the part of the recipient against alloantigens located within the transplanted organ. AR takes two forms: (1) Acute cellular rejection (ACR), in which cytotoxic T lymphocytes and other inflammatory cells invade the renal parenchyma; and (2) Antibody-mediated rejection, which is defined by the presence of donor specific antibodies, morphologic evidence of acute injury and histologic evidence of an antibody-mediated process (*e.g.,* detection of C4D staining in the allograft).

Since most patients with AR are asymptomatic, routine and frequent monitoring of the sCr as a functional measure of allograft function is mandatory in order to detect injury at the earliest possible time. This strategy is flawed for a number of reasons as rising sCr cannot differentiate between the many etiologies of post-transplant injury such as drug-induced nephrotoxicity, BK viral nephropathy or recurrent disease. Furthermore, a rising sCr is a relatively late marker of rejection as a significant amount of histologic damage that may already have been sustained by this time. This point is emphasized by the detection of subclinical rejection on protocol or surveillance biopsies. In subclinical rejection, histologic evidence of rejection is present on a biopsy specimen without elevation of sCr[6,7]. Many studies have demonstrated an association between subclinical rejection on protocol biopsy and adverse graft outcomes. In an analysis of 833 protocol and 306 clinically indicated biopsies, the presence of persistent inflammatory infiltrates correlated significantly with long-term function in the transplanted kidney, independent of an increased sCr[8].

Currently, histologic analysis of tissue obtained by renal biopsy remains the standard for distinguishing AR from other causes of allograft dysfunction only. Renal biopsies are generally considered safe with risk of graft loss around 0.03%. But the procedure carries some inherent risks because of its invasive nature[9,10] including bleeding resulting in ureteric obstruction or development of arterio-venous fistulas, peritonitis, graft loss and even death. In addition, sampling error can occasionally occur since rejection is often a patchy process[11,12]. Furthermore, the financial costs of the procedure are not trivial, averaging about $3000[13].

Thus many investigators have focused their attention on developing non-invasive methods to help in the diagnosis of AR, in particular looking at the measurement of urinary or circulating biomarkers. Identification of reliable non-invasive biomarkers for allograft injury could render invasive monitoring unnecessary. Moreover, rapid and accurate diagnosis could lead to prompt treatment that improves the chances for allograft survival. Some have reasoned that since AR is characterized by lymphocyte and inflammatory cell infiltration of the interstitium and tubules, molecular events occurring in the kidney might be reflected chemically in the urine, even offering more specificity than plasma biomarkers. In fact, some investigators suggest that the transplanted kidney may act as an "*in-vivo*" flow cytometer, sorting cells involved in rejection into the urine[14]. Early studies took an untargeted approach, using multiplex screening assays of urine samples from patients diagnosed with AR to identify chemokines and cytokines elevated in the rejection patients compared to stable controls[15]. Once segregated, targeted assays of these candidate biomarkers were then sought in the urine of transplant patients diagnosed with other causes of graft dysfunction to see if those markers alone or in combination could distinguish AR from antibody mediated rejection,

borderline rejection, BK viral nephropathy, acute tubular necrosis, chronic allograft nephropathy, and stable graft function.

Several potential urinary biomarkers have since been identified and quantified and, as expected, are molecules primarily involved in the major effector pathways of immune mediated cell death. This review inspects the potential role for these noninvasive urinary biomarkers as early indicators of allograft rejection and considers their possible application in distinguishing between acute and subclinical rejection.

NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN AND KIDNEY INJURY MOLECULE-1

A number of urinary biomarkers have been investigated as predictors of and for diagnosing acute kidney injury (AKI) in the general population. But researchers have not fully examined whether they would be useful for distinguishing AR for other kinds of kidney injury. Neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1), previously described in AKI in native kidneys, have shown the greatest potential in this clinical application. NGAL, an innate anti-bacterial factor, is found in activated neutrophils in response to various tubular injuries. Both urine and serum concentrations show an early rise in non-transplant patients in response to AKI. Although NGAL assessment soon after transplant has shown utility in predicting delayed graft function and 3-mo recovery, current evidence suggests that NGAL measurement, at least individually, cannot help distinguish the etiology of acute graft dysfunction. Increased levels of urinary and serum NGAL have also been found in patients with other causes of allograft dysfunction, including calcineurin-inhibitor toxicity, obstructive nephropathy, subclinical tubulitis and infection. Elevated levels of KIM-1, a transmembrane protein expressed on the apical membrane of proximal tubular cells in response to epithelial injury and differentiation, have been shown to predict the need for dialysis in hospitalized patients with AKI of the native kidneys. In transplant recipients with AKI, KIM-1 in the urine provides some information for predicting the rate of decline in renal function, but it cannot distinguish between different kidney pathologies. These findings are not surprising, given the non-specific response of these urinary markers to a variety of tubular injuries[16-19].

URINARY PERFORIN, GRANZYME B AND FAS-LIGAND MRNA

Apoptosis effected by cytotoxic T lymphocytes, thought to play a major role in renal allograft rejections, is mediated by two major effector pathways: The Fas-Fas ligand lytic pathway (Fas-L) and the perforin/granzyme B (GRB) degranulation pathway[20]. Perforin, secreted by cytotoxic effector cells, causes cell death by knocking holes in target-cell membranes. GRB induces DNA fragmentation and cell death by activating caspase 3. Li *et al*[21] looked at whether measuring urinary cell levels of perforin and GRB mRNA could be used to diagnose AR noninvasively. They reported that urinary cell levels of perforin and GRB mRNA were highly accurate in predicting AR (perforin mRNA sensitivity 83%, specificity 83%; GRB mRNA sensitivity 79%, specificity 77%) when compared to stable controls. Yannaraki *et al*[22] used quantitative PCR assay of mRNA from these cytotoxic molecules in addition to Fas-L in kidney transplant recipients with graft dysfunction. The study subjects not only included those with AR but also patients with clinical complications common in kidney transplantation such as urinary tract infections (UTIs), cytomegalovirus infection or disease, chronic allograft nephropathy, and delayed graft function (DGF). mRNA levels of all three molecules were significantly higher in AR than in subjects who showed no clinically evident signs of complication. However, perforin, GRB and Fas-ligand gene expression also seemed to be up-regulated in clinical settings other than AR, including UTI, CMV infection, chronic allograft nephropathy and delayed graft function. For these reasons, this set of biomarkers when used individually would appear to have limited value as noninvasive markers of AR since they are not specific enough in clinical settings to replace the need for biopsy.

To investigate this issue further, Heng *et al*[23] pooled the data from 16 studies including 680 subjects to investigate how well GRB and perforin perform diagnostically as predictors of AR. Similar to the results above, neither GRB nor perforin, evaluated individually, performed reliably as non-invasive diagnostic markers of AR in the clinical setting. However, combining these urinary biomarkers yielded a higher test performance than either biomarker individually. The probability of developing AR increased to 73% from 15% when both GRB and perforin were positive, but was only 2% when both were negative, suggesting that the combined evaluation of GRB and perforin may increase the likelihood of detecting AR in order to conduct earlier therapeutic intervention.

URINARY FOXP3 MRNA

Regulatory T cells (Tregs) play a critical role in maintaining T cell homeostasis under a variety of immunologic conditions. They inhibit autoreactive immune response activation, help maintain self-tolerance and homeostasis of the gut's microbial flora, and promote the immunogenic escape of cancer cells[24-26]. Phenotypically, Tregs are identified as a $CD4^+$ T cell subpopulation that expresses CD25 and cytotoxic T-lymphocyte antigen 4 on their cell surfaces and releases suppressor cytokines interleukin (IL)-10 and IL-35, suggesting a suppressor

role for these cells[27]. San Segundo *et al*[28] suggested that Tregs may help antagonize the inflammatory state associated with kidney transplantation and may be considered a prognostic factor of graft outcome and long-term graft survival. They found that patients who maintained high levels of circulating Treg cells in peripheral blood at 6 and 12 mo post transplantation demonstrated better graft survival at 4 and 5 years follow-up.

Tregs express FOXP3 (the X-linked forkhead/winged helix transcription factor) plays an important role in Treg cell differentiation and function[29]. In fact, *FOXP3* gene mutations result in an autoimmune disease marked by polyendocrinopathy and enteropathy that is fatal early in life[30]. FOXP3 has been examined in many studies analyzing the possible role of Tregs as a potential biomarker for immunologic monitoring in acute T-cell mediated rejection. Recent studies suggest that urinary FOXP3 mRNA levels may offer a noninvasive test to help predict AR and improve outcomes for renal transplant patients. Using quantitative PCR, Muthukumar *et al*[31] measured urinary FOXP3 levels in patients with graft dysfunction and biopsy-proven AR, in patients with stable allograft function and normal allograft biopsy, and in patients with chronic allograft nephropathy. Urinary FOXP3 mRNA levels were higher in the AR group than in the other 2 groups. There were significant inverse relationships between FOXP3 mRNA levels and sCr measured during an episode of AR and between the urinary FOXP3 levels and the time from kidney transplantation to the development of AR. In addition, urinary FOXP3 mRNA levels were significantly higher in the group with successful reversal of rejection than in the group without reversal. Combined FOXP3 transcripts and sCr levels had a better predictive value for reversal of rejection (90% sensitivity and 96% specificity) than either FOXP3 transcripts or Scr alone (90% sensitivity and 73% specificity, 85% sensitivity and 90% specificity, respectively). In addition, patients with AR and high levels of urinary FOXP3 responded better to steroid treatment and had significantly lower risk for graft failure.

The reported incidence of DGF after deceased-donor kidney transplantation has increased despite progress in AR treatment[32]. DGF, defined as the need for dialysis within seven days of transplantation, is associated with an increased incidence of AR and a 40% decrease in long-term graft survival. Between 1985 and 1992, United States transplant patients with both DGF and AR had a 5-year survival rate of 35%[33]. Between 1988 and 2007, incidence of AR in patients with DGF was 49% compared to 35% in non-DGF patients, according to a meta-analysis of 34 studies[34].

In response to data on unfavorable graft outcomes with DGF, Aquino-Dias *et al*[35] examined the expression of perforin, GRB, Fas-L, serpin proteinase-inhibitor 9 (an endogenous GRB blocker) and FOXP3 in peripheral blood monocytes, urinary cells and surveillance kidney biopsies taken from patients with DGF complicated by either AR or acute tubular necrosis (ATN). Expression of all analyzed transcripts was significantly higher in patients with AR than in patients with ATN. FOXP3 provided the highest sensitivity and specificity, as well as positive and negative predictive values (between 94% and 100%). These researchers concluded that mRNA analysis of the genes involved in the alloimmune response in patients with DGF can provide an accurate molecular signature for use in the diagnosis of AR.

CXCL9, CXCL10, AND CXCL11

A number of chemokines are produced during an episode of AR, suggesting their possible use as a urinary biomarker. CXCR3-binding chemokines CXCL9 (monokine induced by gamma interferon, MIG), CXCL10 (interferon gamma-induced protein 10, IP10) and CXCL11 (Interferon-gamma-inducible protein IP-9) are important signaling molecules for recruiting alloantigen-primed T cells to the site of the inflammation and for enhancing pro-inflammatory cytokine production. These chemokines are secreted by leukocytes in the transplant kidney and by tubular epithelial cells. They induce, maintain and amplify inflammatory and immune reactions[36]. CXCL9 and CXCL10 as urinary chemokines to screen for AR were first described *in vitro* by Hancock *et al*[37]. This study showed that acute rejection in heart transplants is accompanied by progressive intragraft production of CXCL9, CXCL10, CXCL11 as well as infiltration of activated T cells with the chemokine receptor CXCR3. The authors demonstrated that $CXCR3^{-/-}$ mice have profound resistance to the development of AR and markedly decreased rates of rejection, concluding that CXCR3 plays a key role in T cell activation and recruitment, and allograft destruction. Thus a rationale may exist for targeting CXCR3 along with conventional immunosuppression in the management of acute allograft rejection.

Subsequent studies have proven a robust association between CXCL10 and the fate of the renal allograft. Tatapudi *et al*[38] investigated the association between the immunohistologic expression of CXCL10 and CXCR3 who underwent diagnostic renal biopsies for graft dysfunction and urinary measurements of CXCL10, CXCR3, and 18S rRNA to determine whether there was a correlation between transcript levels and renal allograft diagnosis. Urinary CXCR3 and CXCL10 mRNA levels were higher in patients with AR than in those without AR. CXCL10 mRNA was found to be 100% sensitive as a marker for AR using a cutoff value of 9.11 copies of CXCL10. Measurement of CXCR3 mRNA had a lower sensitivity (63%) for AR but a higher specificity (83% *vs* 78%) than a CXCL10 assay that used a cutoff value of 11.59 copies. Immunohistologic analysis of allograft biopsies showed prominent CXCL10 and CXCR3 expression during AR, both were absent in stable allografts[38].

Subclinical tubulitis (SCT) has been associated with the later development of IFTA and diminished graft survival. Given that the detection of SCT before permanent graft injury is critical for optimizing graft

outcomes, Schaub *et al*[39] investigated the extent to which concentrations of urinary CXC-receptor 3 (CXCR3) chemokines (*i.e.,* CXCL4/9/10/11) and CCL2 related to subclinical tubulitis. Using ELISA, they measured the levels of CXCL9, CXCL10 and CXCL11 as well as two urinary biomarkers of tubular injury (urinary NGAL and alpha-1 microglobulin) and compared them to two other chemokines (CXCL4 and CCL3) selected as controls in patients scheduled to undergo a protocol renal biopsy. All participants demonstrated stable renal allograft function and an estimated glomerular filtration rate (eGFR) > 40 mL/min and underwent scheduled biopsies at 3 and 6 mo post-transplant or when clinically indicated. Protocol biopsies exhibited normal tubular histology, subclinical borderline tubulitis or subclinical tubulitis, as well as clinical tubulitis Banff 1A/1B or IFTA. Urinary CXCL9 and CXCL 10 were significantly higher in subjects with subclinical tubulitis 1a/1b than subjects with borderline subclinical tubulitis or normal tubular histology. The authors showed that urinary CXCL9 and CXCL10 concentrations correlated closely with the extent of SCT while no distinction was seen for urinary CXCL4/CXCL11/CCL2 and tubular injury markers, suggesting an important role for CXCL9 and CXCL10 as urinary biomarkers of early rejection.

Matz *et al*[40] examined the role CXCL10 as a screening marker for AR. They retrospectively analyzed urinary CXCL10 mRNA and protein expression samples from transplant recipients diagnosed with a Banff Ⅰ-Ⅲor borderline rejection and compared them to samples from patients with UTIs, CMV infection and from control patients. The mean urinary level of CXCL10 mRNA expression was significantly higher in patients with biopsy-proven Banff Ⅰ-Ⅲ or borderline rejection compared to control patients with stable graft function. The difference in CXCL10 expression between control patients and patients with UTI and CMV was not significant.

The investigators also calculated creatinine clearance by the Cockcroft-Gault equation at 3 and 6 mo post-transplant to determine whether elevated urinary CXCL10 expression might predict impaired graft function after 3 and 6 mo defined as GFR < 45 mL/min per 1.73 m^2. They found that urine levels of CXCL10 during the first month post-transplant were significantly higher in patients with impaired graft function than in patients with GFR > 45 mL/min per 1.73 m^2. As a result of these findings, they proposed that urinary *CXCL10* gene and protein expression in renal transplant recipients is upregulated at an earlier time than indicated by renal biopsy, suggesting that CXCL10 is a sensitive marker for ongoing rejection within the transplant kidney despite normal sCr values. They also demonstrated that elevated mean CXCL10 levels in the first month after transplant can predict impaired graft function even in the absence of AR. As such, CXCL10 and its receptor CXCR3 may make attractive targets for therapeutic intervention with chemokine antagonists or receptor blocking agents.

Ho *et al*[41] further described the diagnostic usefulness of urinary CXCL10 as a noninvasive marker of tubulitis, examining urine samples from patients who had renal biopsies done per protocol or for clinically relevant reasons. The investigators separated the subjects into six groups according to histologic findings: normal histology; IFTA; IFTA with borderline tubulitis; borderline tubulitis; subclinical tubulitis; and clinical tubulitis. Urinary CXCL10 accurately discriminated between tubulitis of any degree and normal renal transplant histology. There was no significant difference in urinary CXCL10 concentrations between borderline, subclinical, and clinical tubulitis groups. The urinary CXCL10 to creatinine ratio (CXCL10/Cr) distinguished borderline, subclinical and clinical tubulitis from normal histology and IFTA. Using a cut-off value of 2.87 ng CXCL10/mmol Cr, the ratio had 81.8% sensitivity and 86.4% specificity to differential normal transplant from subclinical and clinical tubulitis. This study validated CXCL10 as a specific marker of active inflammation and confirmed CXCL10 as a noninvasive, sensitive and specific marker for tubulitis[41].

Researchers have also sought to apply these findings to pediatric transplant recipients. A cross-sectional analysis by Jackson *et al*[42] evaluated urinary CXCL9 and CXCL10 in pediatric and adult renal transplant patients. They collected urine from 110 adults and 46 children representing healthy volunteers, stable renal transplant recipients, and recipients with clinical or subclinical AR or BK infection, calcineurin-inhibitor toxicity or IFTA. Urinary CXCL9 and CXCL10 were elevated in children and adults with AR or BK infection but not in subjects with calcineurin-inhibitor toxicity, isolated IFTA, or in healthy controls and stable transplant patients. This study suggests that these chemokines are elevated in intra-graft lymphocytic inflammatory conditions but not in non-inflammatory circumstances. Both urinary CXCL9 and CXCL10 had greater sensitivity and specificity for detecting AR and BK infection than sCr. In addition, CXCL9 and CXCL10 were significantly elevated in the subclinical rejection and subclinical BK infection groups compared with stable patients, but was equivalent to patients diagnosed with BK infection and nephropathy.

The researchers performed a separate pediatric subset analysis to account for different sCr dynamics observed in children. The authors also found a significant difference among study groups with elevated CXCL9 and CXCL10 found in AR and BKI compared to all other patients. As in previous studies, these chemokine assays showed greater sensitivity and specificity than did sCr, but neither chemokine distinguished between AR and BK infection. These data confirm that urine chemokine monitoring identifies patients with renal allograft inflammation. The assay is not a specific diagnostic test for rejection, but it may be useful as noninvasive tool for distinguishing those allograft recipients requiring closer observation from those with benign clinical course[42].

In a recent prospective multicenter validation study conducted through the Clinical Trials in Organ Transplantation-01 protocol, researchers collected 2000

urine samples from 280 adults and pediatric primary kidney transplant recipients[43]. Real-time PCR and ELISA assays were performed on urine sediment to compare urinary mRNAs and proteins representing a number of candidate biomarkers previously reported as elevated during AR. The study stratified patients on the basis of the risk for developing AR or progressive renal dysfunction. Study participants included children, recipients of living donor kidney transplants and African American with low pre-transplant peak panel reactive antibody and negative flow cytometry crossmatches at transplantation. Urine was collected at the time of biopsies performed for clinical indications and by protocol at implantation and at 6 mo. The study found a positive predictive value for predicting rejection of only 61% and 67% for urinary GRB and CXCL9 mRNA respectively, insufficient replace diagnostic biopsies. There was no diagnostic added benefit from combining GRB and CXCL9 mRNA as opposed to CXCL9 mRNA alone. Urinary CXCL9 protein was better than urinary CXCL9 mRNA; combining CXCL9 protein and CXCL9 mRNA provided the best positive (71.4%) and negative (92.5%) predictive values for diagnosing or ruling out AR. Moreover, urinary CXCL9 protein was elevated 30 d before AR was detected clinically, indicating that CXCL9 protein may detect intra-graft inflammation/subclinical injury before renal dysfunction occurs. While urinary CXCL9 protein levels decrease after rejection is treated, further work is needed to confirm whether this is clinically significant.

This study also found that low urinary CXCL9 protein in patients with renal dysfunction strongly correlates with the absence of AR or infection. Urinary CXCL9 was collected at six months post-transplant, with patients grouped according to whether they were at high *vs* low risk for developing late graft dysfunction. The absence of urinary CXCL9 at 6 mo post-transplant defined the subgroup at low risk for development of immune injury. There was a significant relationship between concentrations of urinary CXCL9 protein obtained at 6 mo post-transplant and GFR, with the absence of CXCL9 identified in patients who preserved stable renal function. This was independent of donor type, recipient age or gender, donor specific antibody at or before 6 mo or 6-mo eGFR. This prospective multicenter study concluded that CXCL9 can be a marker for excluding AR with low CXCL9 indicating low immunological risk that may predict stable long-term allograft function.

In another recently published prospective multicenter clinical trial, Suthanthiran *et al*[44] collected 4300 urine specimens from 485 kidney-transplant recipients from day 3 through month 12 after transplantation. Investigators formulated a three-gene signature of CXCL10 mRNA, 18S ribosomal RNA, CD3ε mRNA to distinguish ACR from other etiologies of graft dysfunction. A receiver-operating-characteristic curve analysis showed an area under the curve of 0.85, which corresponded to a 79% sensitivity and 78% specificity in discriminating between those biopsies that showed acute cellular rejection and those that did not show rejection. The diagnostic signatures were not associated with UTIs, blood infection or CMV infection but the values in this profile were also elevated in patients with polyomavirus type BK infection. Additionally, the signature distinguished acute cellular rejection from acute antibody-mediated rejection and borderline rejection. Of note, among patients who developed biopsy-proven rejection, there was a sharp rise in the gene signature in the weeks before rejection[44].

A follow-up study by the same authors built on previous work using urinary mRNA-based signatures to differentiate ACR and AMR from other causes of allograft dysfunction. They collected 52 urine samples from 52 patients with biopsy-proven AR (26 with ACR and 26 with AMR) and 32 urine samples from 32 patients with acute tubular injury without rejection. By using a stepwise quadratic discriminant analysis of mRNA measurement, they identified a linear combination of six mRNAs (CD3ε, CD105, TLR4, CD14, complement factor B, and vimentin) that distinguishes AR from acute tubular injury. In addition, in patients diagnosed with AR, a linear combination of a five-gene signature consisting of mRNAs for CD3ε, CD105, CD14, CD46 and 18S rRNA distinguished ACR from AMR with a cross-validated estimate of the AUC of 0.81. Of note, the two transcripts CD3ε mRNA and 18S rRNA measured in both studies were significantly associated with ACR on biopsy. Therefore, the incorporating these urinary cell mRNA profiles into clinical practice may reduce the need to biopsy patients with acute allograft dysfunction[45].

MIRNA AS A NOVEL BIOMARKER OF ACUTE RENAL ALLOGRAFT REJECTION

In the past decade, research into the role of noncoding RNAs (miRNAs) has substantially increased. miRNA are endogenous, single-stranded molecules made up of around 22 noncoding nucleotides. They act as key regulators of B-and T-cell differentiation, maturation and proliferation and play a role in regulatory T cell function and antigen signaling. They are characteristically very stable in urine samples, in formalin-fixed tissues and highly resistant to freeze-thaw cycles. Their role in regulation of pathological processes, their relative tissue specificity and their presence in biological fluids have triggered translational research into the potential utility of miRNAs as noninvasive biomarkers[46].

Anglicheau *et al*[47] first analyzed the expression of miRNAs in biopsy specimens of renal tissue and in circulating mononuclear cells in patients with AR biopsies. They quantified the intra-graft expression levels of miRNA 142-5p, miR-155, miR 223, miR-10b, miR 30a-3p and let-7c and found that miRNA-142-5p, -155, and -223 are overexpressed in AR biopsies and highly expressed in peripheral blood mononuclear cells. In contrast, miRNA-30a-3p, miR-10b, and let-7c are highly expressed in human renal epithelial cells. Their study

Table 1 Review of the described studies

Ref.	Event	Urinary markers	*n*	Endpoints
Li *et al*[21]	AR	Perforin, GRB	*n* = 151	Potential to predict AR
Yannaraki *et al*[22]	AR	Perforin, GRB and Fas-L	*n* = 162	Levels are increased in different clinical settings (AR, UTI, CMVi or CMVd, CAN, DGF)
Heng *et al*[23]	AR	GRB and Perforin	*n* = 680	Combined use of GRB and Perforin may lead to a better prediction of AR
Muthukumar *et al*[31]	AR	FOX-3mRNA	*n* = 83	Reversal of acute AR and lower risk of graft failure with high levels of FOXP3 mRNA
Aquino-Dias *et al*[35]	AR with DGF	Perforin, GRB, PI-9, Fas-L and Foxp-3 mRNA	*n* = 48	Urinary Foxp-3 with 100% sensitivity and 100% specificity for AR
Schaub *et al*[39]	Subclinical tubulitis	CXCL9/CXCL10, a-microglobulin/Cr, NGAL/Cr	*n* = 88	CXCL9/CXCL10 potential noninvasive biomarkers for subclinical tubulitis
Matz *et al*[40]	AR and prediction of short and long-term graft function	IP-10 mRNA and protein	*n* = 76 for IP-10 mRNA	Incidence of AR: Urinary IP-10 protein observed 2/3 d prior to biopsy with 71% sensitivity and 95% specificity
			n = 100 for IP-10 protein	Long term graft function: Urinary IP-10 predictive of GFR at 6 mo post-transplant
Ho *et al*[41]	Subclinical and clinical tubulitis	CXCL10:Cr	*n* = 102	CXCL10:Cr sensitivity of 73.3% and specificity of 72.7%
Jackson *et al*[42]	AR	CXCL9/CXCL10	*n* = 110 adults and 46 children	Elevated CXCL9/CXCL10 identified AR and BKI
Hricik *et al*[43]	CTOT-1: AR	Urinary protein and mRNACXCL9/CXCL10, GRB mRNA	*n* = 2095	CXCL9 protein with high NPV 92% CXCL9 detects subclinical tubulitis Stratification of patients with low *vs* high risk for future injury Utility of CXCL9 for ruling out acute rejection
Suthanthiran *et al*[44]	AR	Urinary mRNA based signatures	*n* = 4300	Three-gene signature of CXCL10 mRNA, 18S ribosomal RNA, CD3ε mRNA distinguish ACR from AMR and even from other etiologies of graft dysfunction
Matignon *et al*[45]	ACR *vs* AMR	Urinary mRNA based signatures	*n* = 84	mRNAs for CD3ε, CD105, CD14, CD46 and 18S rRNA may be able to differentiate between ACR and AMR
Lorenzen *et al*[48]	AR	miRNAs: miR-10a, -10b, -210	*n* = 88	Low Urinary miR-210 during AR Urinary miR-210 predict outcome of renal transplant Urinary miR-210 novel biomarker of AR

GRB: Granzyme B; PI-9: Proteinase inhibitor-9; NGAL: Urine neutrophil gelatinase-associated lipocalin; Cr: Creatinine; DGF: Delayed graft function; AR: Acute rejection; UTI: Urinary tract infection; CAN: Chronic allograft nehropathy; CTOT-1: Clinical trial of transplantation-1; IP-10: Interferon-g–inducible protein-10; BKI: Polyomavirus BK infection; ACR: Acute cellular rejection; AMR: Antibody mediated rejection; Fas-L: Fas-Fas ligand lytic pathway.

suggested that the altered intragraft expression of miRNAs had cellular basis, and proposed using miRNA expression as a biomarker of renal allograft status.

Urinary miRNA not only shows potential as a novel marker for detecting AR, but may also help predict outcomes in renal transplant patients with AR. In one of the first clinical evaluations of urinary miRNA in patients with AR, Lorenzen *et al*[48] isolated pooled RNA in urinary samples from patients with AR, stable controls without rejection, patients before and after rejection and patients with UTIs. They studied the value of urinary miRNA in predicting long-term outcomes for renal transplant patients with AR. They found that miR-10a, miR-10b and mi-R210 were downregulated in urine samples collected during AR. After successful treatment for rejection, miR-210 expression increased to stable levels. Furthermore, low levels of urinary miR-210 were significantly associated with a decline in GFR at one year after transplantation. Consequently, urinary miR-210 may serve as a novel biomarker for AR and in predicting allograft outcome.

CONCLUSION

Acute rejection carries great significance for renal allograft outcomes, including irreversible allograft dysfunction and on overall graft survival. While non-invasive urinary biomarkers are currently not used in the clinical setting, this review of the literature suggests that they may have a significant role as clinical tools to detect early AR and predict graft survival (Table 1). Unfortunately, there have been few clinical trials to validate the potential biomarkers identified so far, and much work still needs to be done to demonstrate their usefulness in clinical practice. The studies reviewed to date involved a limited number of patients, did not all have robust controls, and did not demonstrate applicability in broad patient populations.

Nevertheless, these noninvasive biomarkers may help not only in facilitating the follow-up kidney function in transplanted patients prior to sCr elevation but also may allow earlier preemptive treatment of AR. An assay for use at home may be helpful in the pediatric population, given challenges in follow up, communication, education and intolerance to routine phlebotomies and biopsies. But they do not yet seem adequate on their own. Thus there may still be a role for renal biopsies. Perforin and GRB mRNA are elevated in clinical settings other than AR such as UTI, CMVi and DGF. Clearly, CXCL9 and CXCL10 are not specific markers for AR as both appeared to be elevated in AR and BKI. Urinary CXCL9 protein has a very high negative predictive value and may be able to detect subclinical tubulitis, permitting earlier therapy and identification of patients at low- *vs* high-risk for future injury. However, the results of this assay do not preclude the need for biopsy for a final diagnosis, particularly if there is evidence of allograft dysfunction. Urinary FOXP3 mRNA may be a helpful tool as a noninvasive marker for the outcome of AR with significantly higher levels in the urine predicting successful reversal of AR and better response in conjunction a diagnostic biopsy. The discovery of urinary cell mRNA-based signatures for the differential diagnoses of acute allograft dysfunction is an exciting development and awaits further validation in independent datasets, particularly in regard to the longitudinal trajectory of the signature and the relationship to diagnostic outcomes. miRNAs haves been described in various renal diseases, including chronic kidney disease, acute kidney injury, and renal cell carcinoma and demonstrate potential as a biomarker for diagnosing AR as well as a predictor of allograft function.

Much work remains to be done on findings ways to predict AR earlier, more accurately, and less invasively than changes in sCr levels and thus improve patient and allograft outcomes. There may still be a role for the renal biopsy as a way of evaluating changes in renal architecture such as increases in fibrosis, and sCr may be still be useful in helping determine renal clearance. But accurate and precise biomarkers to identify AR earlier and less invasively represent a tremendous step forward to improve allograft survival and patient outcomes by allowing treatment for rejection to start immediately upon detection of those biomarkers. These findings serve as the basis for further work to use urinary biomarkers to guide treatment decisions aimed at improving kidney transplant outcomes. Protocols would thus have to be developed for scheduled urinary biomarker evaluation.

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