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Genomic and Proteomic Fingerprints of Acute Rejection in Peripheral Blood and Urine

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Abstract

Acute dysfunction of a kidney transplant can be the result of many different etiologies and an allograft biopsy is frequently necessary to diagnose acute rejection. This invasive procedure, while generally safe, is time consuming, costly and inconvenient. We summarize recent advances in genomic and proteomic techniques using peripheral blood and urine for the diagnosis of acute rejection. While much progress has been made, validation of these new molecular tests in the clinical setting is still required.

Keywords

rejection; kidney; transplantation; gene; transcript; microRNA

Introduction

Acute rejection (AR) is a significant cause of allograft loss and frequently contributes to chronic allograft dysfunction. Rejection may be either cellular or antibody mediated. The detection of acute rejection, regardless of etiology, is critically dependent on measurement of serum creatinine, an insensitive measure of renal injury (1). Ultimately, a kidney biopsy is the gold standard but is invasive, is limited by sampling error and subject to inter-observer variability of the histopathology (2, 3). Thus, sensitive and less invasive methods would be clinically useful for detection of AR. Moreover, while such methods may be diagnostic, it would also be beneficial if they can also be used to monitor immunologic activity prior to established disease. In this way, treatment could be instituted prior to any fixed and irreversible damage to the kidney.

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A number of strategies have been already been outlined in this issue as potential biomarkers of immune activation and alloimmunity. A critical focus over the last decade has been on using “omics” technologies. These technologies include measurements, on a much larger data scale, of biological molecules that have critical roles in the structure and function of an organism. Several approaches in medicine have included the metabolome (study of chemical processes of an organism), the proteome (study of proteins structure and function) and the genome (study of genetic material of an organism). These large scale assessments can be obtained not only in biopsy tissue but on other bodily fluids such as blood and urine, resulting in an opportunity to develop noninvasive assessments of an organism and their immune response to an organ transplant. In this review, we will summarize approaches utilizing key “omics” technologies in solid organ transplantation, focusing on primarily the detection of acute rejection. We will address these studies based on sample location although a combined approach using multiple methods and biological specimens may ultimately emerge as the approach of choice.

Genomic Approaches to the Diagnosis of Acute Rejection (Table 1)

Urine Genomics

Detection of donor reactive T-lymphocytes that are critical in mediating organ rejection may be a potential assay target as a noninvasive strategy. The product of the kidney that is most proximate in terms of detecting the immune response in the allograft is the urine. Cellular elements in the urine can reflect immunologic activity in the kidney allograft. Specifically, these cells can be isolated and gene transcripts isolated and analyzed using semi-quantitative polymerase chain reaction (PCR). This strategy has been exploited by Suthanthiran and colleagues who initially evaluated the expression of granzyme B and perforin, products of cytotoxic T-lymphocytes, in the sedimented pellet from urine samples of recipients with cellular AR (4). In this study of 22 renal-allograft recipients with a biopsy-confirmed episode of cellular AR, levels of mRNA encoding these 2 cytotoxic proteins were significantly higher than in the urine from 63 recipients without evidence of AR.

Further study by this group also found increases in urinary pellet mRNA expression for serine proteinase inhibitor-9 (PI-9), a natural antagonist of granzyme B (5). Here, 29 patients with biopsy-proven AR had higher mRNA PI-9 levels than in and 58 recipients without AR. These levels were also predictive of Banff type II or higher acute rejection changes. Furthermore, PI-9 mRNA expression predicted subsequent graft function at 6 months ($r=0.43$, $P=0.01$). Moreover, this group also reported that mRNA for CD103, a cell-surface marker expressed on alloreactive $CD8^+$ T lymphocytes is also diagnostic of AR with a sensitivity of 59% and a specificity of 75% (6).

Similarly, Kotsch et al, in a small study of 35 patients followed for up to 3 months post-transplant, found that urinary mRNA expression of granulysin, a molecule expressed by cytotoxic T cells, was also diagnostic of AR, with a higher degree of sensitivity and specificity than other cytotoxic effector molecules including granzyme B, perforin, FasL, CD3, or cytokines such as tumor necrosis factor-alpha (TNFα), RANTES, IL-2, IL-10, interferon-gamma (IFN-γ), and transforming growth factor-beta (TGF-β) (7). Specifically, granulysin mRNA was increased in 11 of 14 patients with AR episodes, and in follow up

studies rising levels were found to precede the onset of AR up to weeks before a rise in serum creatinine was observed.

Another approach has been focused on mRNA expression for chemokines and their receptors and the relationship of expression to AR. Tatapudi et al. found urinary cell mRNA levels of interferon inducible protein-10 (IP-10, also known as CXCL10) and its receptor CXCR3 were elevated in AR but importantly were absent in recipients with stable graft function. This elevated expression could be used to distinguish the development of inflammation prior to clinical AR (8). Specifically, of 82 recipients studied, 24 had AR with increased expression of urine mRNA IP-10 ($P < 0.0001$) or CXCR3 ($P < 0.0001$) predicted AR. Receiver-operating characteristic (ROC) curve analysis showed that AR could be predicted using IP-10 with a sensitivity of 100% and a specificity of 78% while CXCR3 had a sensitivity of 63% and a specificity of 83%, making it a bit less optimal.

Additional studies using this methodology have linked upregulated gene expression of other molecules to the presence of rejection. These include the transcription protein FOXP3, a key regulator of regulatory T cell (Treg) development and function. Here, higher levels of urine mRNA for FOXP3 were associated with improved response to rejection treatment and moreover, were associated with lower likelihood of graft loss at 6 months post-transplant (9). This study involved a single transplant center, with urine specimens from 36 subjects with acute rejection, 18 subjects with chronic allograft nephropathy, and 29 subjects with normal biopsy. Other molecules associated with AR include NKG2D protein found on human NK cells and $CD8^+$ T – lymphocytes that acts a receptor for stress-regulated molecules encoded by the major histocompatibility complex class I chain-related (MIC) and UL-16-binding protein (ULBP)/retinoic acid early transcript (RAET) gene family (10) and TIM-3 a membrane protein preferentially expressed on terminal differentiated Th1 cells but not naïve T cells (11).

Considerable landmark work in the use of urinary mRNA as a diagnostic tool, have come through the Clinical Trials in Organ Transplantation (CTOT) consortia. These studies have involved prospective sample collection in multiple recipients from multiple centers, using a variety of immunosuppressive strategies. In the CTOT-04 study, 4300 urine specimens were collected from 485 kidney transplant recipients at timed intervals and at the time of clinically indicated biopsy. A three gene signature that included CD3s, IP-10 and 18s ribosomal RNA was reliably diagnostic of acute rejection (12) with an area under the curve (AUC) of 0.85 (95% CI 0.78-0.91, $p<0.001$). Moreover, this signature was also able to distinguish antibody mediated rejection from cellular rejection with an AUC of 0.78 (95% CI 0.68-0.89, $p<0.001$) and was unaffected by nonspecific inflammation such as urinary tract infection. Importantly, the authors found that there were significant changes in this signature up to 20 days prior to biopsy-proven diagnosis of AR, suggesting that this test could be predictive of impending rejection and hence a reliable test to be done at intervals to detect future rejection episodes. In a related study, Matignon and colleagues studied 52 urine samples from recipients with antibody or T cell mediated rejection. They found that a six-gene signature from a combination of CD3s, CD105, TLR4, CD14, complement factor B and vimentin mRNAs was able to distinguish the two rejection types, and moreover, discriminate from acute tubular injury that was not rejection related with an area under the

curve of 0.92 (95% CI 0.86-0.98) and 0.81 (95% CI 0.68-0.93) respectively (13). However, this transcript signature was not tested to be predictive of future rejection episodes.

Urine microRNA

The study of microRNAs (miRNAs), small non-coding RNAs of 21 to 25 nucleotides that regulate the expression of various cellular proteins by translational repression or RNA degradation by binding to target sites in the 3′-untranslated regions of protein-coding transcripts, have yielded new mechanistic insights into the initiation, modification and progression of numerous human disease. Initial array analysis to detect *soluble* urinary miRNA have identified miR-21, miR-200c, miR-423, and miR-4640 associated with acute kidney injury (AKI) (14). In a larger cohort of AKI subjects (N=98), 27 of which had kidney allografts and biopsy proven tubular injury without rejection, and comparing to another cohort without AKI (n=97), these miRNAs demonstrated a cross-validated ROC curve of 0.91 for diagnosis of AKI.

Work by Lorenzen and colleagues demonstrated a different pattern for AR (15). In this study, 62 recipients had 68 biopsies demonstrating AR, with 55 of these episodes classified as “subclinical AR”. Reduced levels of urine miR-210 was strongly associated with these episodes, compared to urines from recipients with stable function (n=19) or stable function with urinary tract infection (n=13). Interestingly, urine miR-210 returned to control levels after treatment (n=7). The AUC for ROC curves was 0.7 ± 0.07 (CI 0.5-0.8; p=0.04). The value of this marker appears purely diagnostic as urine levels returned to control after treatment. It should be noted that the vast majority of rejection episodes in this study (n=55) were subclinical meaning that they were obtained at a time of surveillance biopsy with no changes in renal function at the time of biopsy. This argues for this test as having potential as a biomarker. However miR-210 levels in urine samples collected *prior* to rejection in only 12 cases were not depressed, suggesting that urine miR-210 cannot predict impending rejection episodes. While these results are quite intriguing, larger numbers of subjects with AR need to be studied for better validation of the findings. Depressed levels of urine miR-210 were also associated with subsequent graft failure, although this association was modest (15). In a study of 125 recipients, investigators could demonstrate a 22-miRNA signature in urine pellets associated with allograft failure and fibrosis (16). This result further suggests the possibility of miRNA in urine pellets as a consideration to monitor for allograft failure.

Foremost, the ability to utilize this “bench-based” test has been hampered by concerns about the ease of performing the assay in spite of the fact that most commercial laboratories have PCR capability as well as the reproducibility of performing the assay. Moreover, there are a multitude of protocols employed that could make data and results sharing difficult when utilizing different lab approaches. Recently, the CTOT consortia core laboratories for molecular biology engaged in a study to evaluate the performance of this assay and to determine if multiple centers using a standardized protocol could obtain similar results (17). In this study, 6 laboratories were provided samples and reagents to isolate RNA perform reverse transcription and quantitative polymerase chain reaction (qPCR) for multiple genes 18S, PRF, GZB, IL8, CXCL9 and CXCL10. All sites were capable of isolating RNA and

performing qPCR and results for all samples and targets highly correlated. All sites could quantify a control sample accurately to within a factor of 1.5. Thus the ability to perform assays on urine and blood specimens with consistent results across multiple laboratories can be practically accomplished and should be taken into consideration for commercialization.

Genomics of blood and its components

Gene transcripts in peripheral blood have also been extensively studied. Both serum and PBMCs have been studies, the latter ideally because lymphocytes infiltrate the allograft and circulating cells may mimic those that infiltrate the allograft during rejection. Moreover, recent studies with additional validation populations are bringing this technique into a clinical reality.

Initial assessment of gene expression in peripheral blood mononuclear cells (PBMC) has focused on specific genes associated with acute rejection. Vasconcellos et al quantified mRNA for perforin, granzyme B and Fas ligand (FasL) in peripheral blood leukocytes (PBLs) of renal transplant recipients in 31 samples (11 with AR and 20 without AR) from 25 renal allograft recipients. For the prediction of acute rejection, perforin mRNA had a sensitivity of 82% and specificity of 75%, granzyme B mRNA a sensitivity of 64% and specificity of 85% and FasL mRNA a sensitivity of 91% and a specificity of 80%. Furthermore, up regulation of any two genes had a positive predictive value of 100% for acute rejection and the absence of up regulation of one or fewer gene had a 95% negative predictive value (18).

Similarly, in work by Dugre et al, mRNA expression of IL-4, IL-5, IL-6, IFN-γ, perforin, and granzyme B in PBMCs were associated with AR when studied in 8 patients with biopsy proven AR and 13 controls without AR. If two or more cytokine markers were up-regulated in a given patient, 75% of the rejecting recipients were identified against 15% of the non-rejecting patients (19). Furthermore, Shoker et al. found that expression of CD154 (CD 40 ligand), a costimulatory molecule expressed on activated T lymphocytes, was elevated in PBMCs of allograft recipients with acute rejection and chronic allograft nephropathy when compared with patients with excellent allograft function and normal controls. Higher levels of CD154 mRNA also correlated with more severe Banff rejection scores (20). In another small study of AR (n=62), Luo et al demonstrated that TIM-3, a molecule highly expressed in activated T lymphocytes, was highly expressed in PBMCs in patients with AR and the ROC AUC was 0.977 (0.92-1.034; p=0.001) but was not predictive of treatment response (21).

In a slightly different focus, Garcia et al studied 19 rejection episodes in 100 kidney transplant recipients. They found that total cell free DNA (tCF-DNA) in plasma was significantly increased in acute rejection often before clinical diagnosis, and returned to baseline after treatment (22). Area under the curve for this assay was 0.925 (0.861-0.965) with the best efficiency at a cut off of 12,000 genome equivalents/mL (sensitivity 89% and specificity of 85%). Other applications of this technology have been to assess donor organ quality (23). To date, this assay has not undergone further validation in transplant recipient populations for the assessment of rejection, in spite of the relative ease of assay and its consistency in performance.

With the evolution of array technology, additional insights have been obtained and a more complex picture is emerging. One of the initial studies assessing the genome in AR was performed by Flechner and colleagues (24). High density DNA chip analysis was performed on peripheral blood lymphocytes (PBLs) and kidney biopsies from normal kidney donors (n=9), from recipients with well-functioning transplants without rejection (n=10), recipients with kidney allograft rejection (n=7), and recipients with allograft dysfunction without rejection (n=5). Rejections were classified as Banff borderline up to Banff IIA. They found a distinct gene signature distinguishing acute rejection, acute dysfunction without rejection and well-functioning transplants with no rejection history. This demonstrated for the first time that array technology could be applied to clinical transplantation and moreover, an opportunity for both noninvasive diagnosis as well as allograft monitoring.

Extensive work by Sarwal and colleagues has provided potential markers that have clinical relevance and may be moving towards the bedside. In a relatively large pediatric study of 367 allograft biopsies and PBMC samples that included recipients with acute rejection (n=115), stable allograft function (n=180), and other allograft injury (n=72), gene microarrays identified a 5-gene set classifier for acute rejection that was subsequently validated on an independent test set (25). This set included *DUSP1*, *PBEF1*, *PSEN1*, *MAPK9*, and *NKTR*, with 83% positive predictive value, 97% negative predictive value and AUC for distinguishing AR from stable function of 0.955. Moreover, such gene transcripts were found useful in other solid organ rejections. Indeed, Li et al. confirmed the ability of this 5 gene signature that is diagnostic of, as well as predictive of acute rejection in recipients with heart allograft rejection (26). The addition of 5 more genes that were detected by quantitative PCR as significantly elevated in the rejection group did not improve the statistical power for detecting rejection.

More recently, Kurian et al prospectively followed 1000 kidney transplants from five different transplant centers and identified patients with biopsy-proven acute rejection (n=63), acute allograft dysfunction without rejection (n=39) and recipients with excellent function and normal biopsy histology (n= 46) (27). Genome wide profiling was done on whole blood from these recipients that were randomly split into two cohorts for discovery and validation. Multiple three-way classifier tools identified 200-highest value probe sets with a positive predictive value of 76% to 95%. This translated into a greater than 90% power at a significance level of $p<0.001$ to use PBMC gene expression to diagnose acute rejection. The authors did not have sufficient participant numbers to test the ability to distinguish between the different sub-types of acute cellular rejection, nor the contribution of antibody mediated injury and felt that while they could diagnose acute rejection, biopsies would still be indicated for histological phenotyping.

Another powerful investigation into the diagnostic capabilities of gene transcripts in peripheral blood utilized meta-analysis of either independent transplant datasets that included 236 biopsies from 4 organs. From this was created a common rejection module (“CRM”) consisting of 11 genes overexpressed in acute rejection in all transplanted recipients. These genes were: BASP, CD6, CXCL10, CXCL9, INPP5D, ISG20, LCK, NKG7, PSMB9, RUNX3, and TAP1. These genes were further tested in another 3 cohorts of transplant recipients for a total of 794 samples. Moreover, in another two independent

cohorts of 151 renal transplant biopsies, the geometric mean of expression the CRM was assessed. In each dataset, the CRM score was significantly higher in the acute rejection group than in stable function group. Each increment of this score increased the odds ratio of acute rejection by 3.63-5.45 and AUC of 0.80-0.83. An incremental increase in score also correlated with the extent of allograft injury on biopsy (28).

The study of the complexity of the immune response using genomics has been further analyzed in a smaller population of recipients (N=10), monitored at frequent intervals, analyzing both whole blood as well as purified cell populations (29). These cells demonstrated markers of activation and proliferation. DNA microarray monitoring revealed cell subset specific changes in functional pathways that were also time dependent. These results indicate the complicated and dynamic immune response to an allograft and further demonstrate the complexity in interpretation of genomic markers for rejection.

miRNA in serum and PBMCs

Similar to the analysis of urine specimens, investigators have studied the expression of miRNA in the serum and PBMC's of recipients with AR as well as in biopsy tissue. In an early study Sui and colleagues examined miRNA in allograft biopsies from 3 patients with acute cellular rejection and compared it with 3 controls. They were able to identify 20 miRNAs that were significantly different in acute rejection (30). Similarly, Angelicheau and colleagues studied miRNA profiles within 33 allograft biopsies (12 AR and 21 normals) and found a strong association between intragraft expression of miRNAs and messenger RNAs and that acute rejection could be predicted using levels of miRNAs (31).

In a small cohort of kidney transplant recipients, (8 with AR and 4 without AR), Betts et al. followed serum miRNA levels serially prior to AR, at the time of AR, and post AR. Samples were obtained from the healthy controls at parallel time points. They determined that miR-223 and miR-10a were significantly depressed at the time of AR compared to serum from these same patients obtained one-year post transplantation. Surprisingly, there was no difference in miRNA levels between patients with AR and control, non-rejection sera (32). These results are interesting but should be reviewed cautiously due to the small size of the participant population but provide again potential further targets for study.

Studies of miRNA expression in PMBCs and acute rejection have not been studied in detail. T cell activation *in vitro* is associated with complex signaling suggest a more complex relationship than previously appreciated. Grigoryev et al. demonstrated 71 differentially expressed miRNAs, 57 of which were previously not known as regulators of immune activation. Specifically, inhibition of miR-155 and miR-221 (novel in T lymphocytes) were associated with T cell proliferation (33). In contrast the expression of miR-142-3p has been associated in operationally tolerant kidney allograft recipients, expressed specifically in B cells, with modulation of nearly 1000 genes in vitro that affect B cell activation (34). In recipients with chronic antibody-mediated injury, miR-142-5p was upregulated in PBMCs as well as in allograft biopsy, but specifically not in a cohort of recipients with AR (35). Further investigation into PMBC expression of miRNA may target miRNAs upregulated in rejection biopsies, as infiltrating lymphocytes may have similar makers when circulating in peripheral blood.

Proteomic Approaches to the Diagnosis of Acute Rejection

Urine proteomics

Concurrent with genomic studies, have been effort evaluation the protein signatures associated with acute rejection. Using a multi-antibody array platform, Srivastava et al identified ANXA11, Integrin α3, Integrin β3 and TNF-α, as candidate proteins whose elevation may constitute a proteomic signature of AR. These proteins were further qualified using Reverse Capture Protein Microarrays (36). This study was done in a small number of transplant recipients (n=40) from a single center, consisting of serially collected samples as well as biopsy tissue. Samples were collected from patients with AR (n=10) and compared to those with stable graft function (n=11) and those with chronic graft injury (n=11) and normal controls (n=8). Further validation of these proteins is needed to determine their utility as markers of acute allograft injury.

Using a non-targeted approach, Hu et al screened urine from 84 recipients with acute allograft injury, as well as 29 with stable graft function and 19 otherwise healthy individuals using an antibody array consisting of 120 chemokines and cytokines. Initially 23 cytokines and chemokines were elevated in acute injury; further confirmation using multiplex bead assay identified urinary interferon gamma induced protein 10kDa (IP-10, also known as CXCL 10) and monokine induced by interferon-gamma (MIG, also known as CXCL9), macrophage inflammatory protein and osteoprotegerin as indicators of acute graft injury (37).

Additional groups have supported the association of urinary chemokines with acute inflammation. Schaub et al. found that levels of CXCL9 and CXCL10 were significantly higher in recipients with tubulitis detected in surveillance biopsies without detectable functional changes (subclinical tubulitis grade 1A/1B, n= 22) when compared with recipients with stable graft function (n=24), subclinical borderline tubulitis (n=18) (38). These investigators further associated these chemokines with enhanced expression of urinary NGAL and alpha-1-microglobulin, both indicative of tubular injury. However, there was no association between subclinical tubulitis and CXCL4, CXCL11 and CCL2. Ho et al. examined CXCL10 using ELISA in urine samples from 91 patients with protocol or indication biopsies. The ratio of urinary CXCL10 to creatinine was able to distinguish borderline, subclinical and clinical tubulitis from normal histology and interstitial fibrosis and tubular atrophy (IFTA) (39). In a combined adult (n=110) and pediatric (n=46) cohort from a single transplant center using a solid phase bead assay, Jackson et al. found that urine CXCL9 and CXCL10 were markedly elevated in acute rejection as well as BK infected allografts but not in allografts with stable function, calcineurin inhibitor toxicity or IFTA (40). Finally, in a larger cohort study of 213 consecutive renal allograft recipients having 362 surveillance biopsies at 3 and 6 months post-transplant as well as 80 indication biopsies within the first year post-transplant, Hirt-Minkowski et al. found that urine CXCL10 correlated well with the degree of tubulo-interstitial inflammation and created a CXCL10 guided strategy to significantly reduce the need for biopsies at their center (41).

While these studies are supportive of specific chemokines associated with rejection, they have typically been samples collected and analyzed retrospectively. In contrast, the Clinical

Trials in Organ Transplantation consortium for project 1 (CTOT-1) conducted a multicenter observational study to specifically identify biomarkers of kidney allograft injury. This cohort consisted of 280 adult and pediatric first kidney transplant recipients. Additional features included the fact that nearly 85% of recipient received antibody induction therapy, and the vast majority received maintenance therapy consisting of a calcineurin agent, mycophenolic acid and steroids. Delayed graft function occurred in 12.5% of deceased donors and *de novo* donor specific antibody occurred in only 11 recipients. The vast majority of allograft biopsies were performed at intervals for surveillance, and 160 biopsies in 99 recipients were performed for cause that included rising serum creatinine or proteinuria. Antibody mediated rejection was uncommon and only 1 case of BK nephropathy was identified. Biomarkers studied included CCR1, CCR5, CXCR3, CCL5 (RANTES), CXCL9 (monokine induced by interferon gamma), CXCL10 (IP-10), IL-8, perforin and granzyme B. Urinary levels of CXCL9 mRNA and CXCL9 protein alone and in combination emerged as being significant in diagnosing and ruling out acute rejection. Importantly, CXCL9 protein and mRNA (negative predictive value 83%) and more so CXCL9 protein (negative predictive value 92%) were useful in serial monitoring of recipients, with a rise in urine protein levels suggesting inflammation. Low levels of urinary CXCL9 protein at 6-month post-transplant in stable function recipients identified individuals at low risk of developing future rejection or drop in renal function (42). Such a marker may be useful in monitoring graft injury and as a helpful strategy for those in which immunosuppression is being tapered or converted.

A more global approach to investigating the proteome has been taken by Sarwal and colleagues using a number of approaches. Sigdel et al used shotgun proteomics with liquid chromatography-mass spectrometry/mass spectrometry and ELISA to analyze 92 urine samples from patients with acute allograft rejection, stable function, recipients with proteinuria and also healthy controls. This extensive analysis identified 1446 urinary proteins specific to transplant recipients. Proteins associated with AR included MHC antigens, complement and extracellular matrix proteins. Tamm-Horsfall protein (also known as uromodulin –UMOD), and CD44 were decreased in urine of recipients with AR and serpin peptidase inhibitor (SERPINF1 or PEDF) was elevated (43). In a follow up study by this group, a study of 50 transplant recipients and 20 controls identified 40 peptides associated with acute rejection. The use of a 6-gene biomarker panel (COL1A2, COL3A1, UMOD, MMP-7, SERPING1, TIMP1) classified AR with high specificity and sensitivity and an area under ROC curve = 0.98) (44).

More recently, these investigators studied a large bio repository of pediatric transplant samples from 262 recipients, consisting of over 2000 samples with matched biopsies (45). 74 recipients had acute rejection, 38 had BK polyomavirus nephropathy, 58 had chronic allograft injury, 8 had nephrotic syndrome, 74 had stable function and 10 were normal controls. Utilizing isobaric tagging for relative and absolute protein quantification (iTRAQ) technology for proteomic discovery with targeted ELISA validation, and utilizing both an exploratory and validation cohort, 9 urine proteins were found to be highly specific for acute rejection from all other diagnoses. There proteins included HLA class II protein HLA-DRB1, Keratin-14 (KRT14), Histone H4 (HIST1H4B), Fibrinogen gamma (FGG), Actin-

beta (ACTB), Fibrinogen beta (FGB), Fibrinogen alpha (FGA), Kerratin-7 (KRT7), and dipeptidy-peptidase-4 (DPP4). Increased levels of FGB, FGG, and HLA DRB1 were validated by ELISA for AR and were segregated from BK infection, suggesting a new surveillance marker for rejection. These results seem quite promising especially since ELISA is a technique already utilized by nearly every hospital lab. However, this patient population due to its pediatric nature is primarily recipients of living donors, with little ischemic injury present and may not be applicable to the general population, and suggest additional testing in more complicated adult patient populations.

Peripheral blood proteomics

The peripheral blood, while perhaps a more stable environment than urine due to absence of circulating proteases, is a challenge for proteomic measurements owing to the extreme range of protein concentrations with abundant plasma proteins constituting 99% of the total protein mass while many proteins of potential interest exist at very low concentrations. In spite of this technical challenge, several groups have tried to utilize serum proteomic strategies to detect and monitor for acute rejection. Freue et al. of the Genome Canada Biomarkers in Transplantation Group used iTRAQ technology to quantitate plasma protein relative concentrations in kidney transplant recipients with and without biopsy proven acute rejection. Of over 300 patients enrolled in prospective monitoring of serum and urine, 27 developed acute rejection and a case control study design was applied using 11 rejectors and 21 controls. They identified a total of 18 plasma proteins involved in inflammation, complement activation, blood coagulation, and wound repair that had significantly different relative concentrations between patient groups. Twelve proteins with a fold-change >1.15 were selected for diagnostic purposes; seven were increased (titin, lipopolysaccharide-binding protein, peptidase inhibitor 16, complement factor D, mannose-binding lectin, protein Z-dependent protease and β2-microglobulin) and five were decreased (kininogen-1, afamin, serine protease inhibitor, phosphatidylcholine-sterol acyltransferase, and sex hormone-binding globulin). Further analysis identified that levels of titin, kininogen-1, and lipopolysaccharide-binding protein could be used to discriminate between AR and controls (46). These pilot data demonstrate complex cell signaling that involves immunity and inflammation, and further study of these in context of the genome is needed in other cohorts to confirm these observations.

Wu et al similarly used iTRAQ labeling and quantitative proteomic technology to examine the serum proteome in recipients in their transplant center. Of 85 rejection episodes in 362 kidney recipients, 5 had plasma available for study compared to 9 others without AR. In this small sample set, 179 proteins were identified of which 66 were at least 2-fold different between rejectors and non-rejectors. Proteins identified were associated with inflammation and complement activation and included transcription factors such as nuclear factor-κB, signal transducer and activator of transcription 1, signal transducer and activator of transcription 3 (47). These results suggested cross talk between the immune response and coagulation pathways and have yet to be confirmed.

Using Luminex™ bead array analysis, Xu et al evaluated the level of 95 cytokines and chemokines and their soluble receptors in sera from 32 recipients with biopsy proven

rejection compared to 38 recipients with stable graft function from a total cohort of 526 recipients collected prospectively over 2 years from 5 transplant centers. The simultaneous quantification of three analytes (IL-1 receptor antagonist, IL-20 and sCD40 ligand) was able to distinguish between acute cellular rejection, delayed graft function and pulmonary infection with 90.9 % sensitivity, 96 % specificity, a positive predictive value (PPV) of 95.2 % and a negative predictive value (NPV) of 92.3 % (48). While these results are interesting and potentially clinically applicable due to the platform utilized, the small subject number studied limits the generalizability of these results.

Conclusion

Much progress has been made in the investigation of genomic and proteomic biomarkers in acute rejection. Several groups have identified signatures that have had internal validation and now await more robust analysis in more complex recipient populations. Indeed, many of the cited studies have focused on single center experiences that lack the racial diversity seen in North American populations. However, identifying such biomarkers is of the utmost and immediate importance to the field, as newer therapeutic agents that may be placed in testing need more sensitive measures of allograft injury. Finally some of these studies have shed light on the immunobiology of transplant rejection and may serve to provide new targets for therapy in and of themselves.

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References

1. Hariharan S, Kasiske B, Matas A, Cohen A, Harmon W, Rabb H. Surrogate markers for long-term renal allograft survival. Am J Transplant. 2004; 4(7):1179–83. [PubMed: 15196079]
2. Williams WW, Taheri D, Tolkoff-Rubin N, Colvin RB. Clinical role of the renal transplant biopsy. Nat Rev Nephrol. 2012; 8(2):110–21. [PubMed: 22231130]
3. Furness PN, Taub N. International variation in the interpretation of renal transplant biopsies: report of the CERTPAP Project. Kidney Int. 2001; 60(5):1998–2012. [PubMed: 11703620]
4. Li B, Hartono C, Ding R, Sharma VK, Ramaswamy R, Qian B, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. N Engl J Med. 2001; 344(13):947–54. [PubMed: 11274620]
5. Muthukumar T, Ding R, Dadhania D, Medeiros M, Li B, Sharma VK, et al. Serine proteinase inhibitor-9, an endogenous blocker of granzyme B/perforin lytic pathway, is hyperexpressed during acute rejection of renal allografts. Transplantation. 2003; 75(9):1565–70. [PubMed: 12792516]
6. Ding R, Li B, Muthukumar T, Dadhania D, Medeiros M, Hartono C, et al. CD103 mRNA levels in urinary cells predict acute rejection of renal allografts. Transplantation. 2003; 75(8):1307–12. [PubMed: 12717221]
7. Kotsch K, Mashreghi MF, Bold G, Tretow P, Beyer J, Matz M, et al. Enhanced granulysin mRNA expression in urinary sediment in early and delayed acute renal allograft rejection. Transplantation. 2004; 77(12):1866–75. [PubMed: 15223905]
8. Tatapudi RR, Muthukumar T, Dadhania D, Ding R, Li B, Sharma VK, et al. Noninvasive detection of renal allograft inflammation by measurements of mRNA for IP-10 and CXCR3 in urine. Kidney Int. 2004; 65(6):2390–7. [PubMed: 15149352]

9. Muthukumar T, Dadhania D, Ding R, Snopkowski C, Naqvi R, Lee JB, et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. N Engl J Med. 2005; 353(22):2342–51. [PubMed: 16319383]
10. Seiler M, Brabcova I, Viklicky O, Hribova P, Rosenberger C, Pratschke J, et al. Heightened expression of the cytotoxicity receptor NKG2D correlates with acute and chronic nephropathy after kidney transplantation. Am J Transplant. 2007; 7(2):423–33. [PubMed: 17173658]
11. Renesto PG, Ponciano VC, Cenedeze MA, Saraiva Camara NO, Pacheco-Silva A. High expression of Tim-3 mRNA in urinary cells from kidney transplant recipients with acute rejection. Am J Transplant. 2007; 7(6):1661–5. [PubMed: 17430399]
12. Suthanthiran M, Schwartz JE, Ding R, Abecassis M, Dadhania D, Samstein B, et al. Urinary-cell mRNA profile and acute cellular rejection in kidney allografts. N Engl J Med. 2013; 369(1):20–31. [PubMed: 23822777]
13. Matignon M, Ding R, Dadhania DM, Mueller FB, Hartono C, Snopkowski C, et al. Urinary cell mRNA profiles and differential diagnosis of acute kidney graft dysfunction. J Am Soc Nephrol. 2014; 25(7):1586–97. [PubMed: 24610929]
14. Ramachandran K, Saikumar J, Bijol V, Koyner JL, Qian J, Betensky RA, et al. Human miRNome profiling identifies microRNAs differentially present in the urine after kidney injury. Clin Chem. 2013; 59(12):1742–52. [PubMed: 24153252]
15. Lorenzen JM, Volkmann I, Fiedler J, Schmidt M, Scheffner I, Haller H, et al. Urinary miR-210 as a mediator of acute T-cell mediated rejection in renal allograft recipients. Am J Transplant. 2011; 11(10):2221–7. [PubMed: 21812927]
16. Maluf DG, Dumur CI, Suh JL, Scian MJ, King AL, Cathro H, et al. The urine microRNA profile may help monitor post-transplant renal graft function. Kidney Int. 2014; 85(2):439–49. [PubMed: 24025639]
17. Keslar KS, Lin M, Zmijewska AA, Sigdel TK, Tran TQ, Ma L, et al. Multicenter evaluation of a standardized protocol for noninvasive gene expression profiling. Am J Transplant. 2013; 13(7):1891–7. [PubMed: 23802725]
18. Vasconcellos LM, Schachter AD, Zheng XX, Vasconcellos LH, Shapiro M, Harmon WE, et al. Cytotoxic lymphocyte gene expression in peripheral blood leukocytes correlates with rejecting renal allografts. Transplantation. 1998; 66(5):562–6. [PubMed: 9753332]
19. Dugre FJ, Gaudreau S, Belles-Isles M, Houde I, Roy R. Cytokine and cytotoxic molecule gene expression determined in peripheral blood mononuclear cells in the diagnosis of acute renal rejection. Transplantation. 2000; 70(7):1074–80. [PubMed: 11045645]
20. Shoker A, George D, Yang H, Baltzan M. Heightened CD40 ligand gene expression in peripheral CD4+ T cells from patients with kidney allograft rejection. Transplantation. 2000; 70(3):497–505. [PubMed: 10949194]
21. Luo Y, Shi B, Qian Y, Bai H, Chang J. Sequential monitoring of TIM-3 gene expression in peripheral blood for diagnostic and prognostic evaluation of acute rejection in renal graft recipients. Transplant Proc. 2011; 43(10):3669–74. [PubMed: 22172823]
22. Garcia Moreira V, Prieto Garcia B, Baltar Martin JM, Ortega Suarez F, Alvarez FV. Cell-free DNA as a noninvasive acute rejection marker in renal transplantation. Clin Chem. 2009; 55(11):1958–66. [PubMed: 19729469]
23. Beck J, Bierau S, Balzer S, Andag R, Kanzow P, Schmitz J, et al. Digital droplet PCR for rapid quantification of donor DNA in the circulation of transplant recipients as a potential universal biomarker of graft injury. Clin Chem. 2013; 59(12):1732–41. [PubMed: 24061615]
24. Flechner SM, Kurian SM, Head SR, Sharp SM, Whisenant TC, Zhang J, et al. Kidney transplant rejection and tissue injury by gene profiling of biopsies and peripheral blood lymphocytes. Am J Transplant. 2004; 4(9):1475–89. [PubMed: 15307835]
25. Li L, Khatri P, Sigdel TK, Tran T, Ying L, Vitalone MJ, et al. A peripheral blood diagnostic test for acute rejection in renal transplantation. Am J Transplant. 2012; 12(10):2710–8. [PubMed: 23009139]
26. Li L, Khush K, Hsieh SC, Ying L, Luikart H, Sigdel T, et al. Identification of common blood gene signatures for the diagnosis of renal and cardiac acute allograft rejection. PLoS One. 2013; 8(12):e82153. [PubMed: 24358149]

27. Kurian SM, Williams AN, Gelbart T, Campbell D, Mondala TS, Head SR, et al. Molecular classifiers for acute kidney transplant rejection in peripheral blood by whole genome gene expression profiling. Am J Transplant. 2014; 14(5):1164–72. [PubMed: 24725967]
28. Khatri P, Roedder S, Kimura N, De Vusser K, Morgan AA, Gong Y, et al. A common rejection module (CRM) for acute rejection across multiple organs identifies novel therapeutics for organ transplantation. J Exp Med. 2013; 210(11):2205–21. [PubMed: 24127489]
29. Grigoryev YA, Kurian SM, Avnur Z, Borie D, Deng J, Campbell D, et al. Deconvoluting post-transplant immunity: cell subset-specific mapping reveals pathways for activation and expansion of memory T, monocytes and B cells. PLoS One. 2010; 5(10):e13358. [PubMed: 20976225]
30. Sui W, Dai Y, Huang Y, Lan H, Yan Q, Huang H. Microarray analysis of MicroRNA expression in acute rejection after renal transplantation. Transpl Immunol. 2008; 19(1):81–5. [PubMed: 18346642]
31. Anglicheau D, Sharma VK, Ding R, Hummel A, Snopkowski C, Dadhania D, et al. MicroRNA expression profiles predictive of human renal allograft status. Proc Natl Acad Sci U S A. 2009; 106(13):5330–5. [PubMed: 19289845]
32. Betts G, Shankar S, Sherston S, Friend P, Wood KJ. Examination of serum miRNA levels in kidney transplant recipients with acute rejection. Transplantation. 2014; 97(4):e28–30. [PubMed: 24531825]
33. Grigoryev YA, Kurian SM, Hart T, Nakorchevsky AA, Chen C, Campbell D, et al. MicroRNA regulation of molecular networks mapped by global microRNA, mRNA, and protein expression in activated T lymphocytes. J Immunol. 2011; 187(5):2233–43. [PubMed: 21788445]
34. Danger R, Pallier A, Giral M, Martinez-Llordella M, Lozano JJ, Degauque N, et al. Upregulation of miR-142-3p in peripheral blood mononuclear cells of operationally tolerant patients with a renal transplant. J Am Soc Nephrol. 2012; 23(4):597–606. [PubMed: 22282590]
35. Danger R, Paul C, Giral M, Lavault A, Foucher Y, Degauque N, et al. Expression of miR-142-5p in peripheral blood mononuclear cells from renal transplant patients with chronic antibody-mediated rejection. PLoS One. 2013; 8(4):e60702. [PubMed: 23577151]
36. Srivastava M, Eidelman O, Torosyan Y, Jozwik C, Mannon RB, Pollard HB. Elevated expression levels of ANXA11, integrins beta3 and alpha3, and TNF-alpha contribute to a candidate proteomic signature in urine for kidney allograft rejection. Proteomics Clin Appl. 2011; 5(5-6):311–21. [PubMed: 21591265]
37. Hu H, Kwun J, Aizenstein BD, Knechtle SJ. Noninvasive detection of acute and chronic injuries in human renal transplant by elevation of multiple cytokines/chemokines in urine. Transplantation. 2009; 87(12):1814–20. [PubMed: 19543058]
38. Schaub S, Nickerson P, Rush D, Mayr M, Hess C, Golian M, et al. Urinary CXCL9 and CXCL10 levels correlate with the extent of subclinical tubulitis. Am J Transplant. 2009; 9(6):1347–53. [PubMed: 19459809]
39. Ho J, Rush DN, Karpinski M, Storsley L, Gibson IW, Bestland J, et al. Validation of urinary CXCL10 as a marker of borderline, subclinical, and clinical tubulitis. Transplantation. 2011; 92(8):878–82. [PubMed: 21876477]
40. Jackson JA, Kim EJ, Begley B, Cheeseman J, Harden T, Perez SD, et al. Urinary chemokines CXCL9 and CXCL10 are noninvasive markers of renal allograft rejection and BK viral infection. Am J Transplant. 2011; 11(10):2228–34. [PubMed: 21812928]
41. Hirt-Minkowski P, Amico P, Ho J, Gao A, Bestland J, Hopfer H, et al. Detection of clinical and subclinical tubulo-interstitial inflammation by the urinary CXCL10 chemokine in a real-life setting. Am J Transplant. 2012; 12(7):1811–23. [PubMed: 22390571]
42. Hricik DE, Nickerson P, Formica RN, Poggio ED, Rush D, Newell KA, et al. Multicenter validation of urinary CXCL9 as a risk-stratifying biomarker for kidney transplant injury. Am J Transplant. 2013; 13(10):2634–44. [PubMed: 23968332]
43. Sigdel TK, Kaushal A, Gritsenko M, Norbeck AD, Qian WJ, Xiao W, et al. Shotgun proteomics identifies proteins specific for acute renal transplant rejection. Proteomics Clin Appl. 2010; 4(1): 32–47. [PubMed: 20543976]

44. Ling XB, Sigdel TK, Lau K, Ying L, Lau I, Schilling J, et al. Integrative urinary peptidomics in renal transplantation identifies biomarkers for acute rejection. J Am Soc Nephrol. 2010; 21(4): 646–53. [PubMed: 20150539]

45. Sigdel TK, Salomonis N, Nicora CD, Ryu S, He J, Dinh V, et al. The identification of novel potential injury mechanisms and candidate biomarkers in renal allograft rejection by quantitative proteomics. Mol Cell Proteomics. 2014; 13(2):621–31. [PubMed: 24335474]

46. Freue GV, Sasaki M, Meredith A, Gunther OP, Bergman A, Takhar M, et al. Proteomic signatures in plasma during early acute renal allograft rejection. Mol Cell Proteomics. 2010; 9(9):1954–67. [PubMed: 20501940]

47. Wu D, Zhu D, Xu M, Rong R, Tang Q, Wang X, et al. Analysis of transcriptional factors and regulation networks in patients with acute renal allograft rejection. J Proteome Res. 2011; 10(1): 175–81. [PubMed: 20812764]

48. Xu X, Huang H, Cai M, Qian Y, Li Z, Bai H, et al. Combination of IL-1 receptor antagonist, IL-20 and CD40 ligand for the prediction of acute cellular renal allograft rejection. J Clin Immunol. 2013; 33(1):280–7. [PubMed: 22948742]

Table 1
Gene Expression Studies in Kidney Transplant Rejection

Study group	Number of subjects	Groups	Gene	Results
Urine				
Li (4)	84	22 AR 63 Controls	Granzyme B Perforin	Both increased in AR
Muthukumar (5)	87	29 AR 58 Controls	Serine proteinase inhibitor-9 (PI-9)	Increased in AR
Ding (6)	79	30 AR 49 other/ SF	CD 103	Increased in AR
Kotsch (7)	35	14 AR 21 no AR	Granulysin	Increased in AR
Tatapudi (8)	82	24 AR 58 no AR	IP-10 CXCR 3	Increased in AR
Muthukumar (9)	83	36 AR 29 normal	FOXP3	Increased in AR
Seiler (10)	109	52 AR 42 no AR 10 UTI 5 CMV 8 ATN	NKG2D	Increased with and prior to AR
Renesto (11)	72	30 AR 30 no AR 12 SF	TIM-3	Increased in AR
Suthanthiran (12)	485	321 for cause/89 surveillance 63 AR 259 No AR	CD3ε IP-10 18s ribosomal RNA	3 gene signature with AR
Matignon et al (13)	84	26 ACR 26 AMR 32 ATN	CD3ε CD105 TLR4 CD14 complement factor B vimentin	6 gene signature with AR
Lorenzen (15)	94	62 AR 19 controls 13 UTI	miR-210	Increased in subclinical AR
Blood and PBMCs				
Vasconcellos (18)	25	11 AR 20 no AR	Granzyme B Perforin Fas ligand	Increased with AR
Dugre (19)	61	8 AR 13 no AR	IL-4 IL-5 IL-6 IFN-γ Perforin Granzyme B	Increased with AR
Shoker (20)	57	20 SF 25 AR or CAN 12 normal controls	CD40L	Increased in AR and CAN
Luo (21)	62	24 AR 20 no AR 18 stable	TIM-3	Increased in AR
Garcia Moreira (22)	100	19 AR 81 no AR	Total Cell-free DNA (tCF-DNA)	tCF-DNA levels with and prior to AR
Flechner (24)	32	7 AR	DNA microarray	Signature with AR

Study group	Number of subjects	Groups	Gene	Results
		8 no AR 8 healthy controls		
Li (25, 26)	367	115 AR 180 SF 72 graft injury from other causes	DNA microarray	5 gene signature with AR
Kurian (27)	148	63 AR 39 graft dysfunction from other causes 46 SF	DNA microarray	Gene signature with AR
Betts (32)	12	8 AR 4 no AR	miR-223 and miR-10a	Reduced with AR

AR = acute rejection, SCR = subclinical acute rejection, SF = stable function, UTI = urinary tract infection, ATN = acute tubular necrosis, CMV = cytomegalovirus infection, BKPVN = BK polyomavirus nephropathy

Table 2
Protein Expression Studies in Kidney Transplant Rejection

Study group	Number of subjects	Types compared	Gene or protein	Results
Urine				
Srivastava (36)	40	10 AR 10 SF 8 normal controls	ANXA11 Integrin α3 Integrin β3 TNF-α	Increased proteomic signature for AR
Hu (37)	132	84 AR 29 SF 19 normal controls	CXCL10 (IP-10) CXCL9 osteoprotegerin	Elevated in AR
Schaub (38)	88	22 SCR 24 SF 17 AR 10 IFTA	CXCL9 CXCL10 NGAL alpha 1-microglobulin CXCL4 CXCL11 CCL2	CXCL9 and CXCL10 increased in SCR, AR
Ho (39)	91 patients	17 SCR 17 AR 22 normal histology 20 IFTA 13 borderline AR	CXCL10	Ratio of CXCL10 to Cr able to distinguish between patient categories
Jackson (40)	110 adults 46 children	SF SCR AR BKPVN IFTA Healthy Controls	CXCL9 CXCL10	Increased with AR and BKPVN
Hricik (42)	280	Surveillance and for cause biopsy	CXCL9 protein/mRNA CCR1 CCR5 CXCR3 CCL5 CXCL10 IL-8 Granzyme B Perforin	CXCL9 protein and mRNA increased with AR
Sigdel (43)	92	AR AF Proteinuria Healthy controls	1446 urinary proteins	Uromodulin and CD44 were decreased and serpin peptidase inhibitor was increased with AR
Ling (44)	70	50 transplant recipients 20 controls	40 peptides, 6 gene biomarker panel	40-peptide panel associated with AR 6 gene biomarker panel associated with AR
Sigdel (45)	262 pediatric recipients	74 AR 38 BKPVN 74 SF 58 chronic allograft injury 10 healthy controls	HLA-DRB1, KRT14 HIST1H4B FGG ACTB FGB FGA KRT7 DPP4	9 urine proteins specific for AR
Serum				
Freue (46)	32	11 AR 21 no AR	Titin kininogen-1 lipopolysaccharide-binding protein	Ratios of these 3 proteins distinguish AR from no AR
Wu (47)	13	5 AR 8 no AR	179 proteins	66 proteins at least 2 fold different between AR and no AR

Study group	Number of subjects	Types compared	Gene or protein	Results
Xu (48)	70	32 AR 38 SF	IL-1 receptor antagonist IL-20 sCD40 ligand	Quantification of these 3 proteins distinguish between AR, delayed graft function and pulmonary infection

AR = acute rejection, SCR = subclinical acute rejection, SF = stable function, UTI = urinary tract infection, ATN = acute tubular necrosis, CMV = cytomegalovirus infection, BKPVN = BK polyomavirus nephropathy