Novel diagnostics in renal transplantation

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Renal transplantation is the treatment of choice for many patients with end stage renal disease. While significant progress has been achieved in short-term outcomes, long-term graft survival has only marginally improved. More than 50% of transplanted kidneys from deceased donors fail within 10 years; and from living donors, within 12 years. A lack of clinical tools to accurately monitor the allograft is a major causative factor in this lack of progress. This paper discusses newly available methods used to assess allograft status with emphasis on the role of circulating chimerism in renal transplantation as a diagnostic indicator for rejection and injury.

Diagnostics in Development

Because of the limitations posed by conventional biopsy procedures, investigators have focused on alternative diagnostic methods based on molecular technology to aid assessment of allograft status. Numerous studies have examined tissue and serum gene expression patterns using both candidate-gene approaches and transcriptional sweeps. Although much of the published work to date has focused on techniques using nucleic-acid based arrays, proteomics and metabolomics offer alternative avenues for the development of novel diagnostic biomarkers of allograft status with the added advantage of identification of potential therapeutic targets.

Candidate gene approach—blood and urine. Several research groups have recently reported the development of urinary and blood RNA markers for allograft rejection. One strategy has been to focus on dysregulation of pathways associated with immunologic stress. This work began by studying concentrations of particular urinary mRNAs including those for perforin, granzyme B, serine proteinase inhibitor 9 (PI-9), CD 103 (natural ligand for E-cadherin), interferon-inducible protein-10 (IP-10) and the chemokine receptor CXCR3 in urinary cells. Several of these gene products have altered expression in cells found in blood, urine, and/or biopsy tissue during rejection episodes. Using this approach for example, Muthukumar et al. demonstrated that urine concentration of FOXP3 mRNA, the transcription factor that denotes graft-protecting regulatory T cells, predicts reversal of acute renal allograft rejection with 90% sensitivity and 73% specificity. Although measurement of individual gene products such as FOXP3 has not replaced conventional biopsies for diagnosis of rejection, detection of such gene products in blood and urine, together with renal function and other immune response markers may be used to generate a “geneprint” useful for diagnosis and clinical management of acute rejection in a renal allograft.

Transcriptome screening. Gene expression profiling has made its way to the field of renal allograft monitoring. Microarrays provide unbiased, simultaneous global expression patterns for more than 40,000 human transcripts. High throughput microarray technology offers a means to study disease-specific transcriptional changes in tissue biopsy, peripheral blood and biofluids.

Much of the available data on gene expression profiling has been obtained using renal biopsy tissue. The key studies have defined a molecular signature of acute rejection, discovered a novel biomarker for chronic antibody mediated rejection, and identified a “superior” molecular score to predict acute rejection in renal biopsy samples. There are also now multiple reports of using peripheral blood samples to monitor allografts such as the “tolerant footprint.” These transcriptional profiles define a peripheral blood biomarker panel of genes associated with operational graft tolerance that has been refined to a cross platform...
combination of biomarkers and bioassays predictive of tolerance in a multicenter European study. Although initial efforts in renal transplantation will be to define triggers for biopsy, in recent related studies in cardiac transplantation, monitoring for rejection with gene-expression profiling using peripheral blood samples was as useful as routine invasive endomyocardial biopsy.

**Proteomics.** The refinement of the techniques that facilitate the study of urinary proteins affords another diagnostic opportunity. Different proteomics approaches have been applied to analyze the urinary proteome in the past, which has helped to build up the list of urinary proteins identified in acute rejection to date. These early studies used gel-based techniques to identify a relatively smaller number of proteins; whereas use of gel-free high performance liquid chromatography and mass spectrometry (“shotgun proteomics”) has proven to be an efficient way to identify a greater number of proteins. A recent report by Sigdel et al. highlights the usefulness of this approach in transplantation.

Using liquid chromatography and mass spectrometry-based techniques to investigate urine from kidney transplant patients, the investigators studied pooled urine samples to identify a unique set of proteins expressed in acute rejection. Three of these proteins Tamm-Horsfall protein (UMOD), Pigment Epithelium-Derived Factor (SERPINF1) and CD44, were further cross-validated by ELISA in an independent set of urine samples, demonstrating significant differences in their abundance in acute rejection. As more discovery studies are performed using similar approaches, the list of potential urine protein biomarkers will expand and require further validation.

**Microchimerism as a Biomarker**

Solid organ transplantation results in large-scale allogeneic chimerism. Carryover of circulating immune and other cells from the graft into the recipient circulation may result in a state of circulating microchimerism. Peripheral blood donor microchimerism was first clinically proposed as facilitating tolerance in liver transplantation, a procedure during which potentially large numbers of immune cells enter the recipient circulation. A few years later in the setting of kidney transplantation, Starzl, et al. also reported donor derived microchimeric cell presence as being associated with donor specific tolerance. However, subsequent studies failed to identify high frequency donor-derived peripheral blood chimerism in recipients of cadaveric kidneys raising doubts as to the importance of this mechanism towards establishing tolerance. These inconsistent results led to a temporary loss of interest in microchimerism as a useful biomarker of tolerance.

It is noteworthy that initial studies of microchimerism in transplantation utilized HLA-based flow cytometry. There are at least two major limitations to this approach. First, even though a large majority of renal transplantation procedures are performed with HLA-discordant donor-recipient pairs, there is a limited panel of available HLA-specific targeting antibodies for use in flow cytometry severely constraining the number of pairs that can feasibly be monitored. The second major limitation is detection sensitivity. Provided a large quantity of recipient blood is available for analysis, the practical detection sensitivity of HLA-based flow cytometry is approximately 1-0.1%. To overcome these limitations, our group and other research teams have developed HLA- and other polymorphism-based quantitative PCR assays capable of targeting unmatched donor-specific alleles at greater sensitivity than flow cytometry techniques. For instance, the HLA-specific quantitative-PCR assays developed by our group are validated for the detection of as little as one allogeneic (donor) genome in a background of 1.2–1.5 x 10^7 host (recipient) genomes. This represents a 150–1,500 fold improvement in sensitivity compared to flow cytometry. Additionally, quantitative PCR sensitivity is greater than that routinely achieved with methods relying on short tandem repeats used to identify blood chimerism after hematopoietic cell transplantation. For the assays we have developed, specificity for target HLA sequences (for both primers and the probe) was verified by testing against genomic DNA derived from a battery of EBV-transformed HLA cell lines. Prior work has demonstrated the use of HLA polymorphism-specific quantitative PCR to quantify allogeneic fetomaternal microchimeric DNA (soluble, cell and tissue-derived) in normal adults and in persons with autoimmune diseases. Because to our knowledge sensitive techniques such as our HLA-specific quantitative PCR have not been applied to study conventional kidney transplantation, persistent donor-derived circulating cellular microchimerism may still prove to be a useful biomarker of tolerance (Fig. 1). In our view, the concept is worth revisiting with this presently available modern methodology.

**Peripheral blood cellular chimerism after combined kidney and hematopoietic cell transplantation.** Lymphohematopoietic chimerism was first described in bovine twins by Owen et al. more than 60 years ago. Individuals who have complete chimerism after myeloablative therapy and bone marrow transplantation subsequently accept solid organ allografts from the same donor. However, myeloablation with this approach is associated with substantial morbidity and mortality. While this may be necessary in patients with hematological malignancy to ensure that all tumor cells are eliminated before bone marrow transplantation, it would not be justified as a treatment strategy in solid organ transplant recipients with otherwise normal hematopoietic systems. Furthermore, hematopoietic cell transplantation across HLA barriers carries a substantial risk of life-threatening graft-versus-host disease. To overcome these barriers, induction of low-level mixed hematopoietic chimerism (the presence of blood cells of both donor and host in origin) following donor bone marrow or peripheral blood infusion concurrent with minimal immunosuppression has been evaluated. Although initial studies failed to demonstrate meaningful microchimerism, three breakthrough articles published in the *New England Journal of Medicine* at the beginning of 2008 highlighted that the induction or development of mixed chimerism in kidney or liver transplant recipients can lead to long-term donor specific tolerance following transplantation, irrespective of whether the chimerism persists. One of these studies by Kawai and colleagues conducted at the Massachusetts General Hospital has achieved long-term acceptance of HLA-mismatched kidney allografts without chronic immunosuppressive therapy.
Chimerism are better tolerated than those matching non-inherited paternal alleles, an effect potentially attributable to tolerance towards persistent maternal microchimerism. Whether identifying prospective ‘pre-tolerant’ recipients to non-inherited maternal allele matched grafts with HLA-specific quantitative PCR detection of maternal microchimerism will predict tolerance is unknown but worthy of future study.

Soluble microchimerism. Generalized renal allograft injury from rejection, infection, drug toxicity and recurrent renal disease may all result in release of soluble nucleic acids of donor origin into blood or urine (see Fig. 1B). Could concentrations of soluble microchimerism be used to non-invasively monitor allograft status? Early studies in blood and urine demonstrated a correlation with soluble concentrations of donor derived DNA with allograft events, however these findings were of limited clinical applicability because PCR methods were based on the detection of Y chromosome sequences (i.e., male donor organs) in female recipients. Our group has begun to revisit this approach with HLA-specific quantitative PCR.

In the first study, HLA-specific PCR was used to identify and quantify concentrations of donor DNA in banked repository sera from patients who had previously undergone simultaneous pancreas-kidney transplantation. There were two major findings. First, for the immediate 7 days following procedure, most grafts shed large amounts of donor DNA into serum suggesting soluble microchimerism is an indicator of harm from ischemia-reperfusion injury. Second, 40-fold higher median concentrations of donor DNA at later times was observed in grafts with biopsy-confirmed acute cellular rejection. In some patients with multiple available serum specimens who harbored high concentrations of donor

![Figure 1.](image-url)
Donor-derived DNA in urine is being used as a biomarker for tolerance and acute allograft rejection. This work was supported by a Norman S. Coplon Extramural Grant from Satellite Healthcare (V.K. Gadi).

Figure 2. Urinary donor HLA concentrations were elevated in the presence of acute cellular rejection (*) and reduced after successful treatment in this patient.

Summary

There are both individual and societal consequences stemming from poor kidney allograft monitoring. At present, a ‘one-size-fits’ all approach is employed for immunosuppression. Immunosuppression is titrated according to clinician judgment based on biopsy, serial creatinine measurements and standard urinary measures. While this strategy has decreased the rate of graft loss from acute rejection events, chronic allograft nephropathy, opportunistic cancers and infections remain common challenges for patients and clinicians. From a societal prospective, the shortage of transplantable kidneys makes the procedure feasible for only a fraction of those who might benefit from the procedure. In this light, it is discouraging that one-fifth of patients waiting for kidney transplantation are previous allograft recipients whose kidney graft has failed from acute rejection or chronic allograft nephropathy. As many as fifteen percent of available donor kidneys are allocated for the purpose of re-transplantation for treatment of graft failure. Improved diagnostics are absolutely necessary to make further gains in kidney allograft longevity.

As the tools described in this review are further refined, it will become ever more necessary to prospectively validate them through what we hope are inter-institutional collaborations. We also suspect that several approaches may be combined to improve the diagnostic accuracy for tolerance and rejection. Our bias is to validate cellular microchimerism as a biomarker for tolerance and soluble donor-derived DNA as an indicator of allograft injury to trigger more intensive investigations with procedures such as biopsy.

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