Biomarkers in Kidney Transplantation

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Contents

Abstract

Kidney transplantation is the optimal renal replacement therapy. The progressions in immunosuppressive drugs improved the short-term survival, but 10-year graft survival is about 50 %, only. Acute or chronic rejection, drug nephrotoxicity, and transplant glomerulopathy all have adverse impacts on graft survival. Most of these events are the result of over- or under-immunosuppression.

On the other hand, tolerance as a state of no immunosuppression in the presence of functioning graft is an ultimate goal of transplantation.

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In order to individualize treatments and recognize the optimal level of immunosuppression, noninvasive methods for diagnosis of acute rejection and tolerance have been developed, and biomarkers in the shade of technological advances would help physician in this way. Peripheral blood cell, plasma, and urine are readily accessible and perfect specimens for identification of biomarkers. This review is focused on recently developed biomarkers in acute rejection and tolerance as the two most important processes in decision-making about immunosuppressive therapy. The clinical utilities and limitations of these markers are discussed in details.

Keywords

Kidney transplantation • Acute rejection • Tolerance • Biomarker • Genomics • Proteomics • miRNA

Abbreviations

AR	Acute rejection
ATI	Acute tubular injury
ATN	Acute tubular necrosis
AUC	Area under the curve
BPAR	Biopsy-proven acute rejection
CAD	Chronic allograft dysfunction
CAMR	Chronic antibody-mediated rejection
CE-MS	Capillary electrophoresis mass spectrometry
CMV	Cytomegalovirus
COT	Clinical operational tolerance
Cr	Creatinine
CXCL-10	C-X-C motif chemokine 10
DGF	Delayed graft function
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
Foxp3	Forkhead/winged helix transcription factor
IF/TA	Interstitial fibrosis/tubular atrophy
IRI	Ischemia-reperfusion injury
IS	Immunosuppression
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MMP-8	Matrix metalloproteinase-8
NPV	Negative predictive value
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PPV	Positive predictive value
qPCR	Quantitative polymerase chain reaction
RT-qPCR	Real-time quantitative polymerase chain reaction
SELDI-TOF-MS	Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry

TCMR	T-cell-mediated rejection
TG	Transplant glomerulopathy
TOL	Tolerance
Treg	Regulatory T-cells
UMOD	Uromodulin
UTI	Urinary tract infection
VEGF	Vascular endothelial growth factor

Key Facts

Key Facts of Operational Tolerance

- Operational tolerance is a state of stable graft function despite cessation of immunosuppressive drug for more than a year without evidences of chronic rejection.
- Most cases were reported in liver transplantation.
- The majority of cases in renal transplantation are due to noncompliance or intentional withdrawal due to lymphoproliferative disorders.
- Lack of donor-specific antibodies and donors of young age are related to operational tolerance.

Key Facts of Costimulatory Signal

- T-cell activation requires two signals.
- Signal 1 is an antigen-specific pathway that involves T-cell receptor and major histocompatibility complex.
- Signal 2 is the result of other T-cell surface receptors and their ligands on antigen-presenting cell.
- Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and CD28 and their ligands B7-1 and B7-2 are the major receptors involved.
- CTLA-4 binding to B7-1 and B7-2 is an inhibitory signal and leads to anergy.
- Abatacept and belatacept are CTLA4Igs that block costimulatory signal.
- CTLA4Ig is a competitive inhibitor of CD28 binding.
- Targeting receptors and/or ligands in costimulatory pathway is a way to increase graft survival.

Definitions

ELISA The enzyme-linked immunosorbent assay is based on antigen and antibody interaction and enzyme-induced color changes in substrate. Antigens are attached into wells in a plate. Then an antibody that can bind to the antigen and is linked to an enzyme is added. The next step is the addition of substrate. The reaction causes color

change in the substrate, and the intensity of the color signal is indicative of the amount of antigen present.

Genomics Genomics is a combination of genome detection methods (polymerase chain reaction) and bioinformatics to detect the whole genome in a cell and to identify the function and pathways that are involved.

Microarray Microarray is one of the tools in genomics, which is consisted of a glass slide with DNA molecules attached to it in specific spots. It detects gene expression, and the data is processed and normalized and the results are expressed in a gene expression matrix. The information from microarray studies is presented either in absolute measures or expression ratio.

MicroRNAs MicroRNAs (miRNAs) are 21–23-nucleotide noncoding RNAs that regulate posttranscriptional gene expression by binding to target mRNA leading to either the degradation of mRNA or inhibition of their transcription.

Proteomics Proteomics is the analysis of the whole protein content of a biofluid. The changes in the proteomes are caused by changes in synthesis or modifications during the course of biologic or pathologic processes. These modifications can be used as specific markers of the process.

SELDI-TOF technique One of the proteomic techniques for profiling the proteome of different types of samples using mass spectrometer. This technique does not need sample preparation procedure and may serve as a diagnostic tool. Low resolution and lack of reproducibility are some of the limitations of this technique.

Introduction

Kidney transplantation is the most physiologic renal replacement therapy. Despite significant improvement in 1-year graft survival, long-term graft survival improvement was minimally increased (Hariharan et al. 2000).

During the early phase (first 2 weeks, mostly) of kidney transplantation, factors affecting the outcome are those related to the status of the donated kidney, ischemia-reperfusion injury, acute tubular necrosis (ATN), and the resulting delayed graft function (DGF). Acute rejections whether antibody-mediated or cell-mediated ones are other determinants of graft survival especially during the first posttransplant year.

During recent years, advances in immunosuppressive protocols lead to better short-term graft survival. On the contrary, the incidence of highly sensitized recipients, extended criteria donors, and marginal kidney quality are rising, and therefore detecting patients at higher risk of acute rejection and prompt intervention is critical to save the organ.

The early-phase insults might occur subclinically and consequently cause chronic allograft rejection, transplant glomerulopathy, and end in chronic allograft loss.

Detecting rejection based on currently available techniques (increased serum creatinine or allograft biopsy) is either inaccurate, late, or invasive.

There is an urgent need for markers of graft status from early to late phase of transplantation to ensure timely diagnosis of events before irreversible histologic damage occurred.

On the other hand, overzealous immunosuppression causes infection and malignancies in the long term. It would be wise to adjust immunosuppressive regimes according to the immunologic risk of each individual patient (Lodhi and Meier-Kriesche 2011).

In order to define a biomarker or a panel of biomarkers for a specific process, apart from accuracy, precision, and validity, one must describe the clinical utility of the marker, such as when to evaluate and the frequency of assessments. Additionally, these biomarkers must be clinically available and cost effective.

Biofluids such as blood and urine are readily available and relatively noninvasive samples with the ability of repeated sampling and follow-up monitoring.

Finding and proving the clinical use of biomarkers of ATN, DGF, acute rejection, transplant glomerulopathy (TG), chronic allograft dysfunction (CAD), and tolerance would help to prolong allograft survival. In the following sections, biomarkers of acute rejection and allograft tolerance will be discussed as a guide for immunosuppression therapy.

Biomarkers of Allograft Rejection

Diagnosis of acute rejection is currently based on histologic assessment of allograft sample, which is invasive and has a minor risk of bleeding complications. Additionally, current markers such as serum creatinine cannot detect subclinical rejections (Rush et al. 1994). To improve clinical outcome, there is a need to find markers that predict events before histopathologic and mostly irreversible evidences of rejection become evident and have the ability to differentiate rejection from other causes of allograft inflammation and dysfunction such as pyelonephritis, viral infection, and ATN.

Differentially expressed proteins in blood or urine sample of transplant patients might help to have early diagnosis, predict outcome, and response to therapy in a noninvasive way.

Urine Biomarkers

Urine is an easily accessible biofluid, which allows repeated sampling and reflects intrarenal processes.

Perforin, Granzyme B, and Fas-L mRNA

The major players in cell-mediated rejection are cytotoxic T-cells. $CD8^+$ T-cells are first cells that appear at the scene of rejection. Activated cytotoxic T-cells release granzyme B and perforin. Perforin allows granzyme B to enter the target cells and lead to cell death via mitochondrial apoptotic pathways. Additionally, a small portion of endothelial cell death is mediated by Fas-ligand (Fas-L) pathway (Choy 2010). Apart from $CD8^+$ T-cells, $CD30^+$ T-cells have been proven to be involved in

alloimmunity, and CD30 acts as a costimulatory molecule (Süsal et al. 2011). Thus, urinary cytotoxic markers might be helpful in diagnosis of acute rejection.

Urinary concentration of perforin and granzyme B mRNA was elevated in 24 patients with biopsy-proven acute rejection (BPAR) compared with 22 patients with other diagnoses (chronic allograft nephropathy, toxic tubulopathy, ATN, and nonspecific findings). The ROC curve for perforin mRNA at the cutoff of 0.9 fg per microgram of total RNA showed 83 % sensitivity and specificity for diagnosis of acute rejection. At the cutoff point of 0.4 fg per microgram of total RNA for granzyme B mRNA, granzyme B had 79 % sensitivity and 77 % specificity in identifying acute rejection (Li et al. 2001). These data demonstrate diagnostic value of cytotoxic markers; however, the question is whether they could distinguish acute rejection from other etiologies of inflammation. In a study, urinary mRNA levels of perforin, granzyme B, and Fas-L were followed longitudinally in 37 cadaveric transplant patients by the means of real-time PCR assay. Urine samples were collected during the episodes of BPAR, cytomegalovirus (CMV) infection and disease, urinary tract infection (UTI), DGF, and CAD. Perforin, granzyme B, and Fas-L mRNA levels were significantly higher in BPAR than controls with stable graft function. Interestingly, the urinary levels of markers were not significantly different among patients with BPAR, UTI, CMV infection or disease, and DGF (Yannaraki et al. 2006). Therefore these markers are not specific for acute rejection and are evidences of graft inflammation.

Granzyme A mRNA

Granzyme A along with granzyme B is the most abundant cytolytic molecules of the effector T-cells. It also triggers inflammation by induction of cytokines. Its role as a biomarker of subclinical and clinical T-cell-mediated rejection (TCMR) has been evaluated in a study on 60 patients in six different groups, including those with stable graft function, CMV infection, calcineurin inhibitor toxicity, subclinical rejection (SCR), TCMR-I (with prominent tubulitis), and TCMR-II (with moderate or severe intimal arteritis and tubulitis). High urinary granzyme A mRNA was able to differentiate patients with SCR and TCMR-I from those stable graft function and calcineurin inhibitor toxicity. However, this marker was also elevated in patients with CMV infection; thus, confronting an increased urinary granzyme A, one must rule out the presence of CMV infection by CMV-PCR (van Ham et al. 2010).

It seems that granzyme A could be a useful marker in diagnosis of subclinical rejection after exclusion of CMV infection and gives the clinician enough time to promptly treat the patients before occurrence of irreversible damage.

Foxp3 mRNA

Regulatory T-cells are known since 1975 and have regulatory role in immune response and are involved in tolerance. In the biopsy samples of acute rejection, increased infiltration of Tregs along with effector T-cells has been shown. The immunoregulatory role of Tregs was proven in acute rejections as they controlled further damage. Forkhead/winged helix transcription factor (Foxp3) is

expressed by Tregs and could be used as a marker of their presence and activity (Brown and Wong 2008).

Urinary expressions of Foxp3 mRNA along with CD3E, perforin, and CD25 were significantly higher in patients with biopsy-proven acute rejection compared with those with chronic allograft nephropathy and stable graft function. Foxp3 mRNA level was inversely correlated with severity of acute rejection. Interestingly, there was no correlation among other markers (perforin, CD3E, and CD25) and serum creatinine in patients with acute rejection. Urinary Foxp3 mRNA was predictive of acute rejection episode reversibility, and at the cutoff of 3.46, it had a sensitivity of 90 % and specificity of 73 % in prediction of reversal of graft function. Furthermore, the combination of serum creatinine and the Foxp3 mRNA level was more accurate in predicting the reversal of acute rejection with 96 % specificity. The results indicate that the higher the Foxp3 mRNA level, the greater the chance of reversal of acute rejection. These are all in line with damage controlling role of Tregs (Muthukumar et al. 2005).

Thus, increased urinary Foxp3 mRNA is useful in diagnosis as well as predicting the outcome of acute rejection.

Cytokine/Chemokine mRNA

Cytokines and chemokines (chemotactic cytokines) play a major role in the inflammatory cascade. Each cytokine represents activation of a specific pathway.

C-X-C motif chemokine 10 (CXCL-10) also known as interferon gamma-induced protein 10 (IP-10) is secreted by monocytes, endothelial cells, and renal tubular and mesangial cells in response to interferon-γ (IFNγ). CXCL-10 by binding to its receptor CXCR-3 on activated T-cells and natural killer cells leads to leukocyte recruitment during acute rejection (Ho et al. 2011).

Data suggested that urinary CXCL-10 elevation preceded serum creatinine rise. Urine CXCL-10 can be used as a marker of inflammation and can distinguish tubulitis (histologic characteristic of cellular rejection) from fibrosis. In a study of 91 patients with a wide range of histologic findings from normal to various degrees of tubulitis (borderline, subclinical, and clinical tubulitis) and those with interstitial fibrosis and tubular atrophy (IF/TA), urine CXCL-10-to-creatinine (CXCL-10/Cr) ratio at the cutoff of 2.87 ng/mmol had 81.8 % sensitivity and 86.4 % specificity in differentiating normal histology from subclinical and clinical tubulitis. At the lower cutoff of 1.97 ng CXCL-10/mmol Cr, the sensitivity and specificity for diagnosis of normal histology versus borderline or subclinical tubulitis were 73.3 % and 72.7 %, respectively (Ho et al. 2011).

Along with CXCL-10, the other CXCR-3 ligand, CXCL-9, was shown to be correlated with subclinical rejection. At the cutoff of 7.5 ng/mmol Cr, CXCL-9 had 86 % sensitivity and 64 % specificity in diagnosis of subclinical tubulitis from normal histology or borderline tubulitis. Urinary CXCL-10 and CXCL-9 were not elevated in those with IF/TA as a sole histologic finding (Schaub et al. 2009).

The advantage of these chemokines is earlier appearance in urine than CXCR-3, perforin, and granzyme B and therefore timely recognition of subclinical tubulitis.

These chemokines have same accuracy in pediatric as well as adult transplant patients (Jackson et al. 2011).

Unlike granzyme B and perforin, urine CXCL-10 level is not increased in other inflammatory processes such as UTI and CMV infection (Ho et al. 2011). Tubulointerstitial inflammation by BK virus and ischemia-reperfusion injury (IRI) might increase urinary levels of CXCL-9 and CXCL-10. Therefore, it is necessary to exclude BK virus infection by plasma PCR. The effects of IRI would not last more than 2 months, and thereafter urine chemokines could be reliable markers of tubulitis due to rejection (Schaub et al. 2009). The influence of UTI on urine chemokines is controversial; thus, to be on the safe side, it is better to rule out UTI by negative urine cultures.

A group evaluated the clinical utility of CXCL-9 in risk stratification, prediction of acute rejection in patients with acute graft dysfunction, and prediction of late graft loss. In the setting of acute graft dysfunction, urinary levels of CXCL-9 mRNA had a negative predictive value (NPV) of more than 92 % in putting acute rejection aside. As its positive predictive value (PPV) was about 61–67 %, this biomarker could not be used instead of the gold standard tissue biopsy, but the high NPV might help to avoid the unnecessary invasive kidney biopsy. The NPV was independent of recipient age, HLA mismatch, and de novo donor-specific antibodies. The elevated urine CXCL-9 mRNA level preceded the serum creatinine increment by almost 30 days, and thus it could be used as a predictor of intragraft inflammation days before the clinically evident increase in serum creatinine and as a guide for prompt treatment. Additionally, high urine CXCL-9 mRNA level at 6 months posttransplantation could predict >30 % decrement in estimated glomerular filtration rate (eGFR) at 24 months posttransplantation. In this study, urinary level of CXCL-9 was higher in patients with acute rejection than in those with BK virus infection (Hricik et al. 2013).

Briefly, the urinary mRNA of CXCL-9 is a promising marker to rule out acute rejection and graft inflammation based on its high NPV. As measurement of CXCL-9 protein by ELISA is easier and more reliable in clinical settings, according to the current data, its use to exclude acute rejection is suggested.

OX40/OX40-L mRNA

During T-cell activation along with T-cell receptor (TCR) and major histocompatibility complex (MHC) interaction on antigen-presenting cells (APC), there are second regulatory signals consisted of costimulatory and co-inhibitory pathways (Fig. 1). The major molecular players of these pathways are from either the immunoglobulin superfamily (CD28, CTLA-4, CD80 and CD86, PD-1, and PD-L) or the TNF family (CD40, CD40L, OX40, and OX40-L) (Ford et al. 2014).

OX40 interaction with its ligand causes memory T-cell generation and cytokine production and results in Th2 response and leads to acute rejection. On the contrary, PD-1 and PD-L ligation acts as an inhibitory signaling pathway on T-cells. In a study, the urinary mRNA expression of costimulatory pathway members was compared between patient with stable graft function and those with biopsy-proven acute rejection. The group reported significantly increased levels of OX40, OX40-L, and PD-1 mRNA in urinary cells of patients with acute rejection. PD-1L levels were not

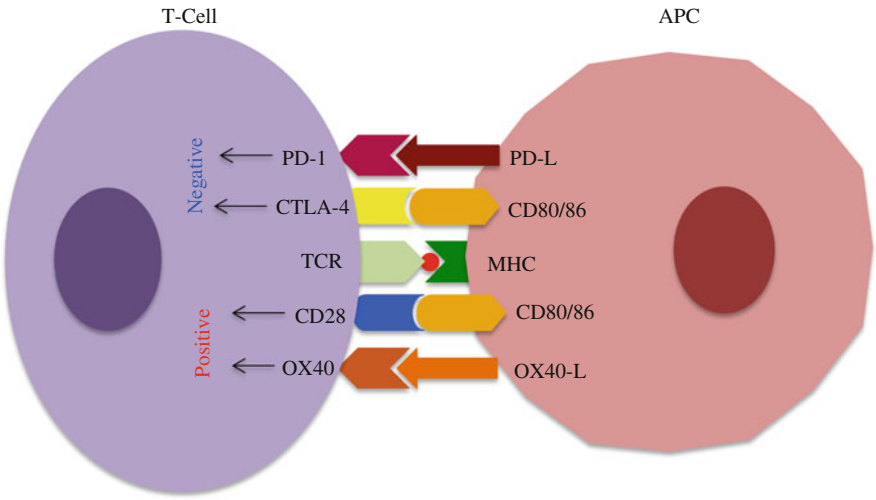

Fig. 1 The costimulatory pathway. Costimulatory signaling results from interaction of ligands on antigen-presenting cells (*APCs*) and the related protein on T-cells. Signals with positive effect lead to T-cell proliferation and cytokine production, and signals with negative effects cause anergy and apoptosis. *CTLA-4* cytotoxic T-lymphocyte-associated protein 4, *MHC* major histocompatibility complex, *PD-1* programmed cell death protein 1, *PD-L* programmed cell death protein 1 ligand, *TCR* T-cell receptor

different between the two groups. OX40 mRNA level alone at a cutoff of 5.98 had a sensitivity of 81 % and specificity of 88 % in diagnosis of acute rejection. When combined with urinary levels of mRNA for OX40-L, PD-1, and Foxp3, the sensitivity and specificity would rise to 95 % and 92 %, respectively. Also the higher OX40-L mRNA level (cutoff value of 3.79) predicted the higher probability of reversal of acute rejection (sensitivity of 69 % and specificity of 100 %) (Afaneh et al. 2010).

Thus, OX40 and its ligand might be used as diagnostic and also predictive biomarker of acute rejection.

mRNA Signature

In a recent study, investigators introduced a urinary mRNA profile instead of a single mRNA in approach to kidney transplant patient with acute graft dysfunction by the means of RT-qPCR. They suggested an mRNA signature with the ability to differentiate acute rejection (AR) from acute tubular injury (ATI).

Combination of urinary values of CD3E, CD105, TLR4, CD14, complement factor B, and vimentin mRNAs formed a diagnostic signature that differentiated AR from ATI. Data suggested that using this signature decreases the unnecessary allograft biopsies. Among patients with AR, a five-mRNA diagnostic model was developed that differentiated acute cellular rejection (ACR) from antibody-mediated rejection (AMR). This model was consisted of CD3E, CD105, CD14, CD46, and 18S rRNA with the area under the curve of 0.81 (95 % confidence interval,

0.68–0.93). Decision curve analysis to assess the clinical benefit was performed in this study (Matignon et al. 2014).

Briefly, using the signature model of mRNAs helps decreasing the number of biopsies in patients with acute graft dysfunction.

Urine miRNAs

MicroRNAs (miRNAs) are 21–23-nucleotide noncoding RNAs that regulate post-transcriptional gene expression by binding to target mRNA leading to either the degradation of mRNA or inhibition of their transcription. They play a role in almost every cellular pathway, and each cell type has its own miRNA pattern. The miRNA profile is representative of the ongoing biologic process and could be evaluated in different biofluids such as urine, blood, and other body fluids. Despite its high cost, RT-qPCR has the ability of detecting a wide range of miRNA when compared with microarray (Mas et al. 2013).

Lorenzen et al. were the first group evaluating the diagnostic role of urine miRNA in acute rejection. Using RT-qPCR, urine samples of 62 patients with biopsy-proven acute rejection were compared with those of patients with stable graft function. The initial data found 21 differentially expressed miRNAs among patients and controls. Among these miRNAs, miR-210 and miR-10b were downregulated, and miR-10a was upregulated in patients with acute rejection compared to the controls with stable graft function. Lower levels of miR-201 were correlated with faster eGFR decline and more severe rejection. Successful reversal of acute rejection normalized the miR-210 and miR-10b levels. The variations in urine levels of miR-210 were independent of the presence of leukocyturia and UTI and age (Lorenzen et al. 2011). If further validation studies confirm these findings, miR-210 could serve as a noninvasive biomarker in diagnosis of acute rejection. However, based on the results from samples collected before evolution of rejection, miR-210 could not predict the impending episodes of acute rejection.

Urine Proteomics

In search for biomarkers, urine proteome profile comes to the center of attention. It is the indicator of local processes in kidney and systemic events that might change urine proteins. In order to characterize urine proteome profile in acute rejection, several studies have been performed (Table 1). Some are discussed in more details.

In a study on 73 patients with graft dysfunction who underwent indication biopsy, by the means of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), two differentially expressed peptides were identified. In patients with acute rejection compared with other causes of graft dysfunction, urinary expression of human β-defensin-1 (HBD-1) was reduced, and urinary expression of α-1-antichymotrypsin (ACT) was elevated. Both of these markers are part of inflammatory and immune responses. When used in combination, the elevated ACT and decreased HBD-1 levels, the sensitivity and specificity for diagnosis of acute rejection would be 85.7 % and 80.2 %, respectively (O'Riordan et al. 2007).

Table 1 Selected urine biomarker for acute allograft rejection

Biomarker	Detection method	Cutoff	AUC	Sensitivity (%)	Specificity (%)	Reference
sHLA-DR	ELISA	15 U/mL	0.88	80	98	Ting et al. (2010)
sUPAR	ELISA	NA	NA	NA	NA	Roelofs et al. (2003)
VEGF	ELISA	3.64 pg/μmol Cr	0.871	85.1	78.4	Peng et al. (2008)
MASP2	LC-MS/MS	NA	NA	NA	NA	Loftheim et al. (2012)
CD103 mRNA	RT-qPCR	8.16 copies/μg Cr	0.73	59	75	Ding et al. (2003)
TIM-3 mRNA	RT-qPCR	1.2[a]	0.96	84	96	Manfro et al. (2008)
ChrY dd-cfDNA[b]	dPCR	≥3 copies of ChrY/K μg Cr	0.80	81	75	Sigdel et al. (2013)

AUC area under curve, *CE-MS* capillary electrophoresis mass spectrometry, *ChrY dd-cfDNA* chromosome Y donor-derived cell-free DNA, *Cr* creatinine, *dPCR* digital polymerase chain reaction, *MASP2* isoform 2 of mannan-binding lectin serine protease 2, *MMP-8* matrix metalloproteinase-8, *NA* not available, *RT-qPCR* real-time quantitative polymerase chain reaction, *sUPAR* soluble urokinase-type plasminogen activator receptor, *TIM-3* T-cell immunoglobulin mucin domain 3, *VEGF* vascular endothelial growth factor

[a]By the relative quantification method $2^{-\Delta\Delta CT}$

[b]It is a sensitive marker for diagnosis of acute allograft injury, but it is not that specific to distinguish acute rejection from BK virus nephropathy

Metzger et al. conducted a multicenter study on 103 transplant patients to identify biomarkers of acute subclinical and clinical rejection and the role of confounding conditions such CMV infection, BK virus infection, and UTI. Capillary electrophoresis mass spectrometry (CE-MS) analyses were used to evaluate urine peptide pattern. Not a single peptide was able to discriminate rejection from other clinical conditions with an acceptable specificity, but a panel of 14 differentially expressed peptides was extracted with the area under the curve (AUC) of 0.89. In order to further validate the panel, the group used it in a validation set and reached an AUC of 0.91 and 93 % sensitivity and 78 % specificity. The presence of UTI and CMV infection did not cause any misclassification. Most of the peptides in this panel were collagen α-1 fragments, which could be an indicator of extracellular matrix degradation and matrix metalloproteinase-8 (MMP-8) activity (Metzger et al. 2011).

Sigdel et al. conducted a shotgun proteomic study with capillary LC-MS/MS on 92 urine samples of patients, including those with biopsy-proven acute rejection, stable graft function, nephrotic syndrome, and healthy controls. The advantage of this study is that they further validated the identified markers by ELISA in an independent set of samples, which is more cost effective, and affordable assay for clinical use. Most of the discriminating proteins in the acute rejection group were MHC antigens, complement pathway proteins, and extracellular matrix proteins. Applying ELISA, they reported significantly decreased uromodulin (UMOD) (AUC = 84.6 %) and CD44 (AUC = 97.3 %) in those with acute rejection with a

Table 2 Urine biomarker panels in diagnosis of acute allograft rejection

Biomarker panel	Detection method	Reference
ANXA11 (↑), integrin α3 (↑), integrin β3 (↑), TNF-α (↑)	Antibody microarrays and reverse capture protein microarray	Srivastava et al. (2011)
IP-10 (↑), MIG (↑), I-TAC (↑)	Luminex assays	Huang et al. (2014)
UMOD (↓), SERPINF1 (↑), CD44 (↑)	LC-MS/MS	Sigdel et al. (2010)
COL1A2, COL3A1, UMOD, MMP-7, SERPING1, TIMP1[a]	LC-MS and multiple reaction monitoring (MRM)	Ling et al. (2010)
HLA-DRB1 (↑), fibrinogen beta (↑), fibrinogen gamma (↑)	iTRAQ	Sigdel et al. (2014)
ID-3796 peptide and 13 collagen α (I, III) fragments	CE-MS	Metzger et al. (2011)
CLCA1 (↑), PROS1 (↑), and KIAA0753 (↑)[b]	2D-LC-MS/MS	Sigdel et al. (2014b)

ANXA11 annexin A 11, *COL1A* collagen type 1 α, *CLCA1* calcium-activated chloride channel regulator-1, *IP-10* IFN-induced protein 10, *I-TAC* IFN-induced T-cell chemoattractant, *MIG* monokine induced by IFNγ, *MMP-7* matrix metalloproteinase-7, *PROS1* vitamin K-dependent protein S, *SERPINF1* pigment epithelium-derived factor (PEDF), *SERPING1* serpin peptidase inhibitor, *TIMP1* tissue inhibitor of metalloproteinase 1, *TNF-α* tumor necrosis factor-α, *UMOD* uromodulin

[a]Gene expression

[b]Exosomal proteins

correlation coefficient of 0.99 and 0.84, respectively, and significantly elevated pigment epithelium-derived factor (PEDF, SERPINF1) levels (AUC = 93.2 %) with a correlation coefficient of 0.78. Thus, this pattern of peptides could verify acute rejection in transplant patients with high sensitivity and specificity independent of age, proteinuria, and immunosuppression protocol (Sigdel et al. 2010) (Table 2).

As there are concerns about the confounding factors such as the amount of proteins in urine (the effect of highly abundant proteins on identification of proteins with lower abundance) and BK virus nephropathy (a pathologically challenging diagnosis), the group conducted a study based on urine peptidomic analysis by LC-MS and multiple reaction monitoring (MRM) on 70 urine samples from 50 transplant patients. Peptidomic analysis provides information about disease-related modification on proteins (proteolytic and antiproteolytic activities). The abundance of UMOD and collagen peptides (COL1A2 and COL3A1) in urine was lower in patients with acute rejection. Evaluating the transcriptome in kidney tissue of these patients demonstrated higher gene expression for matrix metalloproteinase-7 (MMP-7), tissue inhibitor of metalloproteinase 1 (TIMP1), and the serpin peptidase inhibitor (SERPING1) in patients with acute rejection. The abovementioned changes were independent of the presence of BK nephropathy. Apart from being a specific biomarker profile, this panel sheds light on the underlying mechanism of injury during acute rejection and subsequent chronic graft fibrosis: the collagen cascade (Ling et al. 2010).

Recently, the isobaric tags for relative and absolute quantitation (iTRAQ) proteomic technique was used to identify biomarkers of acute rejection. The proteins then were validated by ELISA. Of a total of 389 measured proteins, nine were highly specific for acute rejection. These were identified as: HLA class II protein HLA-DRB1, keratin-14 (KRT14), histone H4 (HIST1H4B), fibrinogen gamma (FGG), actin-beta (ACTB), fibrinogen beta (FGB), fibrinogen alpha (FGA), keratin-7 (KRT7), and dipeptidyl-peptidase-4 (DPP4). These markers could differentiate acute rejection from chronic allograft injury and BK virus nephropathy. Further validation, by ELISA in independent samples, showed increased urinary levels of HLA-DRB1, fibrinogen beta, and fibrinogen gamma (Sigdel et al. 2014a).

Overall, urine peptidomics and proteomics are raising horizon in the land of biomarker studies. The identified profile needs to be validated by a less time and cost-consuming technique such as ELISA for routine clinical utility.

Blood Biomarkers

Evaluating blood biomarkers is also a minimally invasive way to diagnose acute rejection. However, the diagnostic profile might be confounded by systemic milieu, and its sensitivity and specificity might decline. Numerous markers were introduced by different studies using various techniques, but clinical validation is needed before routine application (Table 3).

Table 3 Selected serum biomarker for acute allograft rejection

Biomarker	Method	Sample	Reference
Granzyme B, perforin, Fas-L	RT-PCR	PBL	Vasconcellos et al. (1998)
Foxp3	RT-PCR	PBL	Aquino-Dias et al. (2008)
IFNγ – producing memory T-cell	ELISPOT	Pretransplant PBML	Nickel et al. (2004)
Nitric oxide		Serum	Bellos et al. (2011) and Masin-Spasovska et al. (2013)
PECAM1	ELISA	Serum	Chen et al. (2010)
HLA class I (ABC)	Flow cytometry	Peripheral blood CD3+/CD8+ T lymphocytes	Tian et al. (2009)
Titin, kininogen-1, and LPS-BP	iTRAQ	Plasma	Freue et al. (2010)
IL-1R antagonist, IL-20, and sCD40 ligand	Luminex™ bead array analysis	Serum	Xu et al. (2013)

ELISA enzyme-linked immunosorbent assay, *ELISPOT* enzyme-linked immunosorbent spot, *IL-1R* interleukin-1 receptor, *iTRAQ* isobaric tagging for relative and absolute protein quantification, *LPS-BP* lipopolysaccharide-binding protein, *PBL* peripheral blood leukocytes, *PBML* peripheral blood mononuclear cells, *PECAM1* platelet endothelial cell adhesion molecule 1

CD30

CD30 as a marker of Th2-type immune response has been shown to be associated with allograft outcome (Pelzl et al. 2002). Soluble CD30 (sCD30) as a potential marker of an alloimmunity reaction was evaluated in 203 living kidney transplant patients before, on the fifth day posttransplantation, and at the time of acute increase in serum creatinine with ELISA kit. sCD30 levels among patients with BPAR were compared with those of patients with stable graft function and non-rejection cause of acute allograft dysfunction (including CMV infection, ATN, and calcineurin inhibitor toxicity). sCD30 level on the fifth day posttransplantation with the cutoff value of 41 U/ml predicted the occurrence of acute rejection in the first 6 months with a sensitivity and specificity of 70 % and 71.7 %, respectively. It could not predict the 2-year graft survival. Pretransplant sCD30 level could not predict acute rejection, and there was a significant elevation in sCD30 level during the episodes of BPAR. Thus, sCD30 level after transplantation and its changes could be used as a predictor of acute rejection (Nafar et al. 2009). In a multicenter study on 2,322 transplant patients, investigators demonstrated an association between day 30 posttransplant CD30 level and 3-year graft survival. CD30 levels $\geq$40 U/ml on day 30 were associated with high anti-HLA antibody activity and could be considered as a marker of alloimmunity (Süsal et al. 2011). Same results were obtained in an earlier study, of course with smaller sample size but longer follow-up of 5 years posttransplantation (Delgado et al. 2009). Thus, posttransplant CD30 level might be utilized as a marker of increased alloimmunity and if proved by clinical trials might be used as a guide to immunosuppressive dose adjustment.

Genomics

In order to enhance the sensitivity and specificity of peripheral blood diagnostic tests, transcriptional profile (genomics) was utilized by the means of microarray studies. Gene expression in peripheral blood samples was extensively evaluated in association with acute rejection. Since 1998 that Vasconcellos et al. described the correlation of cytotoxic lymphocyte gene expression (perforin, granzyme B, and Fas-ligand) and acute rejection (Vasconcellos et al. 1998), there are a wide range of studies evaluating gene expression of various effector molecules in diagnosis and prediction of rejection.

T-cell immunoglobulin mucin domain 3 (TIM-3) is a membrane glycoprotein expressed on Th1 cells, cytotoxic T-cells, natural killer cells, and Th17. It has a known role in inducing tolerance. TIM-3 binding to its ligand, galectin-9, results in reduction of cytotoxicity of $CD8^+$ T-cells. TIM-3 mRNA level is proposed as a biomarker of effector T-cell activation and was evaluated in 24 patients with acute rejection, 20 patients with ATN, and 18 patients with stable graft function by the means of RT-PCR. Peripheral blood cell TIM-3 mRNA was significantly higher among patients with acute rejection, and this increased level was not due to decreased GFR. At the threshold of 1.58, TIM-3 mRNA had 100 % sensitivity and 87.5 % specificity in discriminating acute rejection from ATN. The TIM-3 mRNA level did not differentiate refractory from responsive acute rejection (sensitivity of 66.7 % and specificity of 57.1 %). Despite encouraging results, a lack

of biopsy-proven acute rejection in all the cases and exclusion of infective causes of impaired renal function (CMV infection, UTI) brings up the need for further validation of the marker (Luo et al. 2011).

In order to bring biomarkers from bench to beside and assessing their clinical utilities and their limitations, recently the gene expression profiles of patients were studied.

In a large cohort, 367 blood samples from pediatric transplant patients, including 115 patients with biopsy-proven acute rejection, 180 cases with stable graft function, and 72 cases with other causes of graft dysfunction (chronic allograft injury, viral or bacterial infection, calcineurin inhibitor toxicity, and borderline acute rejection), microarray analysis and subsequent quantitative PCR led to the discovery of a five-gene panel. This gene panel consisted of DUSP1, MAPK9, NKTR, PBEF1, and PSEN1. The gene profile is representative of immunologic activity and injury: leukocyte recruitment; B-cell, T-cell, and monocyte activation; oxidative stress; apoptosis; IL-2 pathway activation; increased adhesion; and vascular smooth muscle cell injury. Except MAPK9 and NKTR, which were under-expressed, the remaining three genes were overexpressed in patients with acute rejection. The data was further validated in an independent cohort.

The five-gene model can discriminate acute rejection from those with stable graft function with a sensitivity of 91 % and specificity of 94 % and a NPV of 97 % (AUC 0.955). It also has the ability to separate acute rejection from other causes of graft dysfunction with 91 % and 90 % sensitivity and specificity, respectively. None of the confounding factors affected the results, and the high NPV in the setting of graft dysfunction might decrease the unnecessary biopsies. The downside of the five-gene profile is its inability in detecting borderline rejection and distinguishing humoral from cellular rejection (Li et al. 2012). Further validation for clinical utility in adult recipients is required.

To validate the five-gene panel (DUSP1, MAPK9, NKTR, PBEF1, and PSEN1) in Korean patients, Lee et al. conducted a study on 143 recipients. Patients with acute cellular rejection had significantly lower levels of MAPK9 and higher PSEN1 than controls. However, patients with acute antibody mediated had the similar profile with controls and those with other graft injuries (BK nephropathy, calcineurin inhibitor toxicity, glomerulonephritis, and ATN). Conversely, PSEN1 level was lower and MAPK9 level was higher in patients with other graft injuries. The two-gene set alone had 73.33 % sensitivity and 75 % specificity (AUC, 0.841) in discriminating acute cellular rejection from other causes of graft injury. However, the five-gene set in combination with clinical variables had 90 % sensitivity and specificity (AUC, 0.964) and PPV of 93.1 and NPV of 85.1. Therefore, this five-gene panel is a promising tool for diagnosis of acute cellular rejection from other causes of graft dysfunction (Lee et al. 2014).

Recently, Roedder et al. studied blood gene expression on 558 blood samples of 436 transplant patients both pediatric and adults in a multicenter study. Using real-time quantitative PCR (RT-qPCR), patients with acute rejection were compared with patients with other causes of graft dysfunction (chronic allograft injury, chronic calcineurin inhibitor toxicity, BK virus infection, and acute tubular nephritis).

They utilized the previously reported ten-gene panel (DUSP1, CFLAR, ITGAX, NAMPT, MAPK9, RNF130, IFNGR1, PSEN1, RYBP, and NKTR) (Li et al. 2012) and added seven genes (SLC25A37, CEACAM4, RARA, RXRA, EPOR, GZMK, RHEB). This 17-gene panel showed a significantly higher sensitivity (82.98 %) and specificity (90.63 %), with an AUC of 0.94 (95 % CI 0.91–0.98, $p < 0.001$). The 17-gene panel identified as the Kidney Solid Organ Response Test (kSORT) was validated in a 124 sample independent cohort, and a further cross-validation was performed on 100 samples. In the validation group, the mean predicted probability of acute rejection was significantly different between the two groups as reported in the training set. The kSORT is a sensitive and specific noninvasive test to detect acute rejection whether cellular or antibody mediated. Its high specificity and NPV (91.58 %) make it a valuable marker with a utility as a negative predictor of rejection. As most of the genes in the panel are related to monocyte activation, and monocyte activation is evident in both cellular- and antibody-mediated rejection, one of the limitations of kSORT is its inability to differentiate between these two types of rejection. In order to be used as a predictor of acute rejection, the group designed a longitudinal multicenter study and evaluated 191 blood samples before, at the time, and after acute rejection in an independent cohort. kSORT could predict clinical acute rejection in more than 60 % of samples up to 3 months before the clinical or histological event. After further validations, this panel might replace the invasive protocol biopsy in prediction of subclinical rejection. The group also created a risk score for acute rejection called kSAS (kSORT analysis suite). kSAS algorithm is able to categorize patients according to the risk of acute rejection: high risk for AR (risk score ≥ 9), low risk for AR (risk score ≤ -9), and indeterminate (risk score <9 and > -9) (Roedder et al. 2015).

It seems that after further validation in clinical trials, kSORT could be used as a diagnostic and predictive marker of acute rejection.

miRNAs

Like urine samples and tissue biopsies, peripheral blood samples could be assessed for the presence of miRNAs with the ability to diagnose acute rejection.

miRNAs were evaluated in 32 renal transplant patients including 11 patients with biopsy-proven acute rejection. Both intragraft and peripheral blood mononuclear cells (PBMCs) were evaluated for miRNA expression. miR-142-5p, miR-155, and miR-223 were overexpressed both in biopsy samples and in the peripheral blood (Angelicheau et al. 2009). The study showed correlation between tissue and serum markers, which could be the base for further investigations.

In a study on 12 transplant patients, eight of which had an episode biopsy-proven acute rejection, expression of miRNAs was analyzed in serum by qPCR. miR-223 and miR-10a were significantly reduced among patients with acute rejection. Although the results are encouraging, they must be interpreted keeping in mind the small number of cases (Betts et al. 2014). On the contrary, Lui et al. in their report on 12 transplant patients with acute rejection (in a cohort of 33 patients) demonstrated elevated levels of miR-223 in PBMCs at the time of rejection with a sensitivity of 92 % and specificity of 90 % in diagnosis of acute rejection

(Scian et al. 2013). Small number of cases and different study design may explain the discrepancies.

In a cohort of 112 transplant patients and 11 healthy controls, the miRNA profile of patients with chronic antibody-mediated rejection (CAMR) differed from that of acute rejection. Increased expression of miR-142-5p in PBMCs has been reported in CAMR. It was also reported to be able to discriminate CAMR from those with stable renal function (AUC, 0.74) (Danger et al. 2013).

As mentioned above, most of the miRNA studies are on urine samples, and the recent data opens new fields in biomarker studies in PBMCs or blood samples.

Overall, biomarker identification is a science in evolution, and there is a long way ahead in order to introduce a biomarker or a panel of biomarkers with accurate clinical utility to substitute the invasive gold standard "allograft biopsy."

Biomarkers of Tolerance

Allograft transplantation is the treatment of choice in patients with end-stage renal disease. The downside of transplantation is the long-term need for immunosuppression with infections, malignancies, and nephrotoxicity of drugs as the main side effects. Tolerance gives the opportunity to cease the immunosuppression or to minimize it. Attempts to induce tolerance were not a great success. In order to identify patients who are candidates for immunosuppression minimization or withdrawal, biomarkers of tolerance have been evaluated among patients with "clinical operational tolerance (COT)." COT is a state of tolerating the allograft in the absence of immunosuppressive drugs without pathologic evidences of rejection for at least 1 year. About 100 patients with kidney transplantation have been reported to be at the state of COT, mostly due to noncompliance or lymphoproliferative disorders (Orlando et al. 2010). The clinicians need an assay to guide them in safe reduction in immunosuppression in selected patients without increasing the risk of acute rejection; thus, most of the studies conducted on patients with COT. To evaluate biomarkers, a sample size of at least 200 is needed, and in order to compensate the lack of adequate sample size, studies were conducted on training set, validation set, and cross-validation sets. Data on urine biomarkers are rare. Most promising data come from gene expression studies in peripheral blood, although flow cytometry and ELISA methods also have been used (Gökmen and Hernandez-Fuentes 2013) (Table 4).

Gene Expression Studies

Gene expression microarray assays using RT-qPCR are valuable tools for biomarker discovery and extracting functional and biological role of the marker by the means of bioinformatics.

One of the earliest studies on biomarkers of tolerance was conducted by Brouard et al. They performed a microarray study on a group of 17 COT patients (5 in

Table 4 Biomarkers of tolerance

Biomarker set	Detection method	Reference
IGKV4-1, IGLL1, IGKV1D-13[a]	Multiplex real-time PCR	Newell et al. (2010)
CD79B, TCL1A, HS3ST1, SH2D1B, MS4A1, TLR5, FCRL1, PNOC, SLC8A1, FCRL2[a]	Microarray, RT-PCR	Sagoo et al. (2010)
Foxp3, CCL20, TLE4, CDH2, PARVG, SPON1, RAB30, BTLA, SMILE, SOX3, CHEK1, HBB, DEPDC1, CDC2[a]	Microarray, RT-qPCR	Brouard et al. (2007)
KLF6, BNC2, CYP1B1[a]	Microarray, qPCR	Roedder et al. (2015)
miR-142-3p	Microarray	Danger et al. (2012)
Urine CD20	RT-qPCR	Newell et al. (2010)

qPCR quantitative polymerase chain reaction, *RT-qPCR* real-time quantitative polymerase chain reaction
[a]Gene set as a biomarker

training group and 12 in test group) and compared the results with healthy controls and those with various graft statuses (chronic rejection, stable graft function on immunosuppressive therapy, and those on steroid monotherapy). A set of 49 genes was identified as the footprint of tolerance. Among these genes, 33 distinguished tolerance from chronic rejection with 86 % sensitivity and 99 % specificity. The identified genes were involved in costimulatory signaling and memory T-cell response. They also suggested a role for transforming growth factor-β pathways. If validated in larger cohorts, this panel could be used as a guide for immunosuppression reduction (Brouard et al. 2007).

In a cohort of 25 COT patients (off immunosuppressive drug for at least a year, 20 due to noncompliance), 33 patients with stable graft function, and 42 healthy controls, a microarray study conducted on whole-blood total RNA. A set of five genes were differentially expressed between COT and stable patients – TUBB2A, TCL1A, BRDG1, HTPAP, and PPPAPDC1B – all of which were involved in B-cell activation. The COT group had higher expression of CD20 transcript in urine sediment compared to those on immunosuppressive drugs. After performing multiplex RT-PCR, a 3-gene set found to predict tolerance – IGKV4-1, IGLL1, and IGKV1D-13 – with PPV of 83 % and NPV of 84 %. Whole-blood flow cytometry confirmed a significantly higher number of total B-cells, naïve B-cells, and transitional B-cells (CD19+CD38+CD24+IgD+) in COT patients than in those with stable graft function on drugs. Among the flow cytometry results, transitional B-cell had the highest predictive value for COT (85 % and 96 % PPV and NPV, respectively). These results pointed out the important role of B-cell in tolerance and introduced the 3-gene set as a predictive marker of tolerance (Newell et al. 2010).

Following this study, Sagoo et al. studied a cohort of 71 kidney transplant patients – 11 patients with COT, 11 patients on low-dose prednisolone only, 40 patients on full immunosuppression, and 9 patients with pathologic evidence of chronic rejection. Interestingly, the COT group had the highest degree of HLA mismatch but undetectable donor-specific anti-HLA antibodies. Microarray, RT-qPCR, and flow cytometry techniques were applied on peripheral blood monocyte cells (PBMCs). Recipients with COT (like healthy controls) had the highest B-cell-to-T-cell ratio, which was the result of an elevated number of B-cells rather than reduction of T-cell population. COT patients had decreased proportion of memory B-cells and activated T-cells and increased proportion of transitional B-cells as previously reported by Newell et al. Additionally, tolerant patients had a high ratio of Foxp3/α-1,2-mannosidase in peripheral blood. The microarray data and RT-qPCR resulted in a 10-gene set with diagnostic capability (See Table 4). The set could discriminate COT from non-tolerant transplant patients with 80.6 % sensitivity, 89 % specificity, and 93 % NPV (Sagoo et al. 2010).

Overall, these studies pointed out the significance of B-cell and natural killer cell (NK cell) expansion in tolerant patients. The B-cell signature of tolerance has been developed in two independent cohorts (increased number of naïve and transitional B-cells), and after further validation in larger cohorts, this could be used to choose patients for immunosuppression minimization or cessation. In a prospective observational study, Viklicky et al. tried to validate the abovementioned gene set as a guide in immunosuppression minimization. They compared operational tolerance-associated transcripts (MS4A1, CD79B, TCL1A, TMEM176B, Foxp3, TOAG-1, MAN1A1 (α-1,2-mannosidase), and TLR5) in patients with and without acute rejection in the first day posttransplantation. The expressions of MS4A1 (CD20), CD79B, TCL1A, and TOAG-1 as markers of naïve and immature B-cells were significantly higher in patients without acute rejection, as well as the Foxp3/α-1,2-mannosidase ratio. The expression of TLR5 was not different between the examined groups, and TMEM176B expression was higher in rejection group (Viklicky et al. 2013).

It seems that these seven genes have the capacity to be used as criteria to select patients who are still on immunosuppressive regimes for drug minimization or withdrawal.

In the most recent study, with the aim of providing a highly cross-validated COT gene signature in blood samples and estimating the frequency of the gene signature in patients on immunosuppressive drug, 571 peripheral blood samples were assessed by microarray, qPCR, and flow cytometric analysis (cross-platform) in a four-stage study design. The smallest gene set with the best performance in detection of COT was a three-gene set, KLF6, BNC2, and CYP1B1, with 84.6 % and 90.2 % sensitivity and specificity, respectively. Besides the strong B-cell signature in tolerance, the flow cytometric analysis results demonstrated decreased total number of T-cells and $CD4^{-}/CD3^{+}$ T-cells in COT patients. On the other hand, monocytes and dendritic cells were significantly increased in COT patients.

The study cross-validated the previous findings and introduced a highly validated assay to recognize patients with tolerance, which are still on immunosuppressive drugs and are targets of immunosuppression reduction. The frequency of predicted accommodation was 7.3 % by this assay (Roedder et al. 2015).

Briefly, all the abovementioned genomic studies and the cross-platform biomarkers of tolerance (gene expression, flow cytometry, anti-donor immune response, and anti-donor antibodies) are the first few steps in the long way of establishing personalized transplantation medicine.

Potential Applications to Prognosis, Other Diseases, or Conditions

Two major treats in the allograft patients are rejection and infection. In order to counteract these treats, balanced immunosuppression is needed. Determining the immunologic risk of every patient, and adjusting the immunosuppressive regime according to it, is the optimal way to face this issue.

In the light of novel biomarkers, physicians will be able to estimate the immunologic risk in the pretransplant period and prescribe initial immunosuppression in a personalized fashion rather than in a protocol-wise manner. Biomarkers of acute rejection after transplantation can be utilized to identify subclinical rejections and provide timely intervention before clinical and yet irreversible histological changes occur. The absence of these biomarkers helps in choosing appropriate patients for immunosuppressive withdrawal in the presence of the tolerance molecular signature.

Both the tolerance signatures and the rejection predictors are beneficial during the follow-up of patients with minimization or cessation of immunosuppressive agents. Figure 2 is a schematic plan of what would be the treatment approach if we have validated and easily performed biomarkers in future.

Summary Points

- This chapter is focused on novel biomarkers in diagnosis of acute rejection and tolerance among kidney transplant patients.
- Subclinical acute rejection could only be diagnosed by protocol biopsy, which is an invasive procedure.
- Biomarkers with the ability to diagnose subclinical rejection and guide therapy might improve long-term graft survival.
- High urinary granzyme A mRNA is able to differentiate patients with subclinical rejection and mild T-cell-mediated rejection from those with stable graft function. But CMV infection must be ruled out.
- Urine mRNA of CXCL-9 with its high negative predictive value is a useful biomarker in excluding acute rejection as the cause of impaired graft function.
- Urine mRNA of Foxp3 and OX40 predict occurrence of rejection.

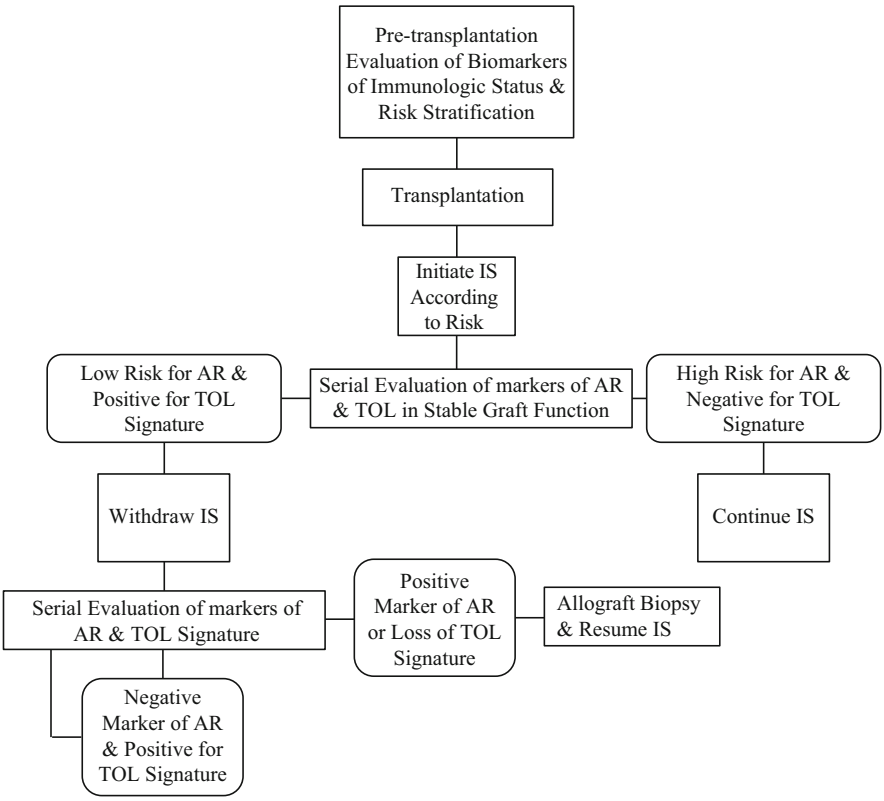

Fig. 2 The proposed clinical utility for biomarkers in individualization of immunosuppressive therapy. With the goal for individualization of IS therapy, biomarkers could be used in pretransplant period to identify high immunologic risk patients in need of strong IS regimes and low-risk patients who might benefit from withdrawal of IS. During the posttransplantation period, biomarkers of acute rejection help recognizing the subclinical rejections. If the patient has the TOL signature and devoid markers of rejection, he/she would be considered for IS withdrawal. After withdrawal serial assessment of biomarkers would be mandatory for timely diagnosis of loss of tolerance and predicting rejection. *AR* acute rejection, *IS* immunosuppression, *TOL* tolerance

- Urine proteomic and genomic (mRNA and miRNA) studies are in the path of evolution and soon be used clinically in predicting and detecting acute rejection.
- Posttransplant serum level of CD30 might be utilized as a marker of increased alloimmunity and a guide to immunosuppressive dose adjustment.
- The 17-gene panel identified as the Kidney Solid Organ Response Test (kSORT) is the best genomic marker of acute rejection till now.
- Increased expression of miR-142-5p in peripheral blood cells is diagnostic for chronic antibody rejection.
- Clinical operational tolerance is a state of tolerating the allograft in the absence of immunosuppressive drugs without pathologic evidences of rejection for at least 1 year.

- The tolerance signature was introduced based on genomic studies.
- Different cross-platform studies identified gene sets for diagnosis of tolerance.
- The identified gene sets pointed out the role of naïve and transitional B-cells and natural killer cells in maintenance of tolerance against allograft.

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