Biomarkers in Disease:
Methods, Discoveries and Applications
Series Editor: Victor R. Preedy

Vinood B. Patel
Victor R. Preedy *Editors*

Biomarkers in Kidney Disease

Springer Reference

Biomarkers in Disease: Methods, Discoveries and Applications

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In the past decade there has been a sea change in the way disease is diagnosed and investigated due to the advent of high throughput technologies, such as microarrays, lab on a chip, proteomics, genomics, lipomics, metabolomics, etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, etc. In many instances these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics and bioinformatics these markers have been used to identify individuals with active disease or pathology as well as those who are refractory or have distinguishing pathologies. Unfortunately techniques and methods have not been readily transferable to other disease states and sometimes diagnosis still relies on single analytes rather than a cohort of markers. Furthermore, the discovery of many new markers have not been put into clinical practice, partly because of their cost and partly because some scientists are unaware of their existence or the evidence is still at the preclinical stage. In some cases the work needs further scientific scrutiny. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the formulation of *Biomarkers in Disease: Methods, Discoveries and Applications*. The series covers a wide number of areas including for example, nutrition, cancer, endocrinology, cardiology, addictions, immunology, birth defects, genetics and so on. The chapters are written by national or international experts and specialists.

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Vinood B. Patel • Victor R. Preedy
Editors

Biomarkers in Kidney Disease

With 208 Figures and 142 Tables

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Volume Preface

In the present volume, ***Biomarkers in Kidney Disease***, we have sections on

- **General Aspects**
- **Circulating and Body Fluid Biomarkers**
- **Specific Diseases and Conditions**
- **Molecular, Cellular, and Histological Variables**
- **Functional and Structural Variables**
- **Resources**

While the Editors recognize the difficulties in assigning particular chapters to particular sections, the book has enormously wide coverage and includes the following areas, analytes, and conditions: osmolal gap, metabolic acidosis, metabolomics, hypoxia, micro-RNAs, creatinine, spot urine markers, neutrophil gelatinase-associated lipocalin (NGAL), chemokines, angiotensinogen, flow cytometry, leucocytes, lymphocytes, exosomes, *N*-acetyl-beta-D-glucosaminidase (NAG), endothelin, methylated arginines, albuminuria, cystatin C, homocysteinemia, fetal beta2-microglobulin, proteinuric biomarkers, apelin, copeptin, BLyS and APRIL cytokines, glutathione transferase, growth arrest-specific protein 6 (Gas6), urokinase receptor, urea nitrogen, allograft damage index (CADI), antibody arrays, malondialdehyde, matrix metalloproteinase-2 (MMP-2), plasminogen activator inhibitor-1 (PAI-1), fibrosis, kidney biopsies, next generation sequencing (NGS), cell-cycle arrest biomarkers, integrin-linked kinase (ILK), molecular biomarkers, M-type phospholipase A2 receptor, ultrasound elastography, aortic pulse wave velocity, renal arterial resistance index, pulmonary pressure, glomerular filtration rates, and erythrocyte width. A wide spectrum of acute and chronic conditions are described including, nephritis, neoplastic disease, transplantation, allograft damage, cystic fibrosis, diabetes, IgA nephropathy, focal segmental glomerulosclerosis, renal microthrombosis, and dialysis.

There are also many other analytes and conditions described within this volume.

Finally, the last chapter is devoted to locating resource material for biomarker discovery and applications.

The chapters are written by national or international experts and specialist. This book is specifically designed for clinical biochemists, nephrologists, specialists working within the field of kidney disease and treatments, health scientists, epidemiologists, and doctors and nurses, from students to practioners at the higher level. It is also designed to be suitable for lecturers and teachers in health care and libraries as a reference guide.

April 2015 Victor R. Preedy
London Vinood B. Patel

Series Preface

In the past decade, there has been a sea change in the way disease is diagnosed and investigated due to the advent of high-throughput technologies and advances in chemistry and physics, leading to the development of microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics, etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, and many other conditions too numerous to list here. In many instances, these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology, immunoassays, or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics, and bioinformatics these markers have been used to identify individuals with active disease as well as those who are refractory or have distinguishing pathologies.

Unfortunately, techniques and methods have not been readily transferable to other disease states, and sometimes diagnosis still relies on a single analyte rather than a cohort of markers. Furthermore, the discovery of many new markers has not been put into clinical practice partly because of their cost and partly because some scientists are unaware of their existence or the evidence is still at the preclinical stage. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the book series **Biomarkers in Disease: Methods, Discoveries and Applications**. It imparts holistic information on the scientific basis of health and biomarkers and covers the latest knowledge, trends, and treatments. It links conventional approaches with new platforms. The ability to transcend the intellectual divide is aided by the fact that each chapter has:

- ***Key Facts (areas of focus explained for the lay person)***
- ***Definitions of Words and Terms***
- ***Potential Applications to Prognosis, Other Diseases, or Conditions***
- ***Summary Points***

The material in **Potential Applications to Prognosis, Other Diseases, or Conditions** pertains to speculative or proposed areas of research, cross-transference to other diseases or stages of the disease, translational issues, and other areas of wide applicability.

The Series is expected to prove useful for clinicians, scientists, epidemiologists, doctors, and nurses, and also academicians and students at an advanced level.

April 2015 Victor R. Preedy
London

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Volume 1

About the Editors

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Dr. Vinood B. Patel B.Sc., Ph.D., FRSC is currently a Reader in Clinical Biochemistry at the University of Westminster and honorary fellow at King's College London. He presently directs studies on metabolic pathways involved in liver disease, particularly related to mitochondrial energy regulation and cell death. Research is being undertaken to study the role of nutrients, antioxidants, phytochemicals, iron, alcohol, and fatty acids in the pathophysiology of liver disease. Other areas of interest are identifying new biomarkers that can be used for diagnosis and prognosis of liver disease, understanding mitochondrial oxidative stress in Alzheimer's disease, and gastrointestinal dysfunction in autism. Dr. Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his Ph.D. in Protein Metabolism from King's College London in 1997. His postdoctoral work was carried out at Wake Forest University Baptist Medical School studying structural-functional alterations to mitochondrial ribosomes, where he developed novel techniques to characterize their biophysical properties. Dr. Patel is a nationally and internationally recognized liver researcher and was involved in several NIH-funded biomedical grants related to alcoholic liver disease. Dr. Patel has edited biomedical books in the area of nutrition and health prevention, autism, and biomarkers and has published over 150 articles, and in 2014 he was elected as a Fellow to The Royal Society of Chemistry.

Victor R. Preedy B.Sc., Ph.D., D.Sc., FRSB, FRSH, FRIPHH, FRSPH, FRCPath, FRSC is a senior member of King's College London (Professor of Nutritional Biochemistry) and King's College Hospital (Professor of Clinical Biochemistry; Honorary). He is attached to both the Diabetes and Nutritional Sciences Division and the Department of Nutrition and Dietetics. He is also founding and

current Director of the Genomics Centre and a member of the School of Medicine. Professor Preedy graduated in 1974 with an Honours Degree in Biology and Physiology with Pharmacology. He gained his University of London Ph.D. in 1981. In 1992, he received his Membership of the Royal College of Pathologists, and in 1993 he gained his second doctoral degree, for his contribution to the science of protein metabolism in health and disease. Professor Preedy was elected as a Fellow of the Institute of Biology (Society of Biology) in 1995 and to the Royal College of Pathologists in 2000. He was then elected as a Fellow to the Royal Society for the Promotion of Health (2004) and The Royal Institute of Public Health and Hygiene (2004). In 2009, Professor Preedy became a Fellow of the Royal Society for Public Health, and in 2012 a Fellow of the Royal Society of Chemistry. In 2015, the Society of Biology received its Royal Charter, so Professor Preedy became an FRSB. In his career, Professor Preedy worked at the National Heart Hospital (part of Imperial College London) and the MRC Centre at Northwick Park Hospital. He has collaborated with research groups in Finland, Japan, Australia, USA, and Germany. He is a leading expert on biomedical sciences and has a long-standing interest in analytical methods and biomarkers, especially their applications to the study of health and disease. He has lectured nationally and internationally. To his credit, Professor Preedy has published over 500 articles, which includes peer-reviewed manuscripts based on original research, reviews, abstracts, and numerous books and volumes.

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Part I

General Aspects

Biomarkers in Kidney Transplantation

1

Mohsen Nafar and Shiva Samavat

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

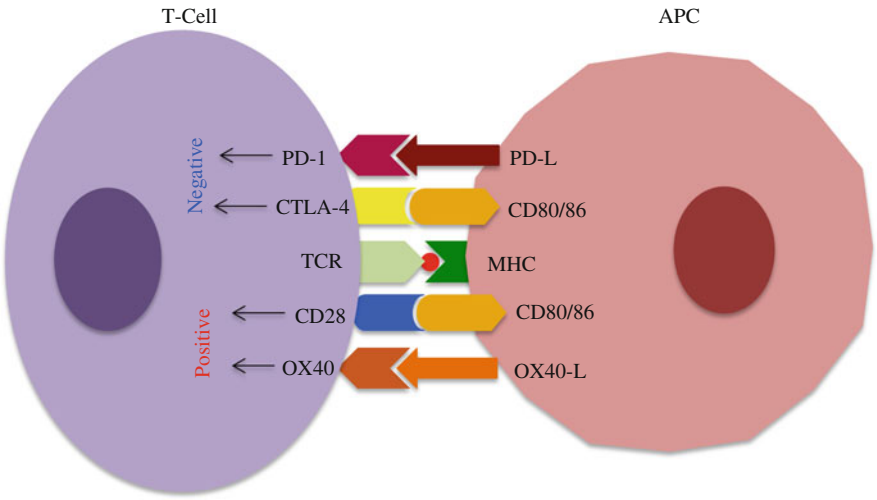

Fig. 1 The costimulatory pathway. Costimulatory signaling results from interaction of ligands on antigen-presenting cells (*APCs*) and the related protein on T-cells. Signals with positive effect lead to T-cell proliferation and cytokine production, and signals with negative effects cause anergy and 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).