INVITED REVIEW

Molecular Assessment of Kidney Allografts: Are We Closer to a Daily Routine?

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Summary

Kidney allograft pathology assessment has been traditionally based on clinical and histological criteria. Despite improvements in Banff histological classification, the diagnostics in particular cases is problematic reflecting a complex pathogenesis of graft injuries. With the advent of molecular techniques, polymerase-chain reaction, oligo- and microarray technologies allowed to study molecular phenotypes of graft injuries, especially acute and chronic rejections. Moreover, development of the molecular microscope diagnostic system (MMDx) to assess kidney graft biopsies represents the first clinical application of a microarray-based method in transplantation. Whether MMDx may replace conventional pathology is the subject of ongoing research, however this platform is particularly useful in complex histological findings and may help clinicians to guide the therapy.

Key words

Kidney transplantation • Gene expression • RT-qPCR • Microarray • MMDx • Molecular microscope

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Introduction

Kidney transplantation is the treatment of choice for end-stage renal disease. Acute and chronic rejection represent the main obstacle for long-term outcomes (Sellares *et al.* 2012, Sis *et al.* 2010). Kidney graft rejection diagnostics was traditionally based on clinical and histological criteria. The Banff classification was introduced to improve and standardise histological diagnostics for transplanted organs (Solez *et al.* 1993). However, histology is descriptive in principle and limited to semi-quantitative assessment only. Therefore, similar morphologies may actually reflect non-homogenous cohorts with different outcomes. A better understanding of the mechanisms behind kidney graft rejection could yield novel preventive, diagnostic, and therapeutic approaches. Transcriptomics is an innovative tool used in several medical disciplines, including transplantation (Halloran *et al.* 2014). Assessment of molecular disturbances has already been incorporated into the Banff classification of kidney allograft pathology (Haas *et al.* 2014, Loupy *et al.* 2017, Haas *et al.* 2018). This review summarises the updated developments on molecular pathology in kidney transplantation.

Limitations of traditional diagnostic tools

Kidney grafts can be affected by nephrotoxicity, infections, vascular pathology, as well as transmitted, recurrent, or de novo diseases, among which the rejection is the most important. Two types of kidney allograft rejection have been recognized, T-cell-mediated (TCMR) and antibody-mediated rejection (ABMR). TCMR and ABMR differ in terms of pathogenesis, pathology, and prognosis, require tailored treatment, and cannot be

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distinguished exclusively based on clinical data (Sa *et al.* 2016). Recently, as the main cause of kidney allograft loss in the long-term follow-up the ABMR has been considered (Sellares *et al.* 2012, Sis *et al.* 2010).

Histologically, TCMR is distinguished from ABMR by the presence of mononuclear tubulitis and interstitial inflammation while microvascular inflammation, arteritis, acute tubular injury or thrombotic microangiopathy are observed in the latter case (Cornell 2016, Haas *et al.* 2018). The presence of complement component C4d in the peritubular capillaries reflects antibody-endothelium interaction (Cornell 2016, Haas *et al.* 2018) in ABMR. However, C4d assay has poor reproducibility (Mengel *et al.* 2013). C4d staining results may vary from negative (which does not exclude ABMR), to diffuse positive, and depend on method used (Cornell 2016, Haas *et al.* 2014, Sis *et al.* 2010). Arteritis and glomerulitis may occur in both ABMR and TCMR (Cornell 2016), or may not be associated with rejection (Cornell 2016). Tubulitis with interstitial inflammation can be a feature of tubulointerstitial nephritis regardless of aetiology. Despite TCMR, tubulointerstitial inflammation may be borderline, although in other patients borderline changes can be benign (Hruba *et al.* 2015). Similarly, double contours are not entirely unique to transplant glomerulopathy (TG) in chronic ABMR, as they also occur in chronic thrombotic microangiopathy and recurrent or de novo immune complex glomerulonephritis (Cornell 2016). Furthermore, kidney grafts can undergo mixed rejection or a combination of several pathologies, especially at a late post-transplant stage.

Histology assessment is greatly influenced by sample size. On the issue of sampling error, the Banff community recommends the examination of two cores containing the renal allograft cortex (Racusen *et al.* 1999). Histological lesions are graded semi-quantitatively using arbitrarily defined grades. Criteria for the diagnoses rely on empirical rules and are the result of consensus (Roufosse *et al.* 2018). All of these factors have led to insufficient inter-observer reproducibility for lesion scoring and diagnosis, potentially resulting in inappropriate treatment (Furness and Taub 2001, Mengel *et al.* 2007, Halloran *et al.* 2014, Broecker and Mengel 2015, Solez and Racusen 2013, Smith *et al.* 2019). The lack of independent validation is another recognised pitfall in the histological Banff classification (Mengel *et al.* 2007).

Potential of molecular diagnostics

Initially, RT-PCR evaluation of single transcripts or subsets of several transcripts associated with T-cell or cytokine burden were performed in small patient cohorts. Studies showed associations of acute rejection with higher mRNA expressions of several cytokines (Krams *et al.* 1992), Toll-like receptors (Dessing *et al.* 2010), as well as of chronic graft injury with profibrogenic cytokines and chemokines (Hribova *et al.* 2007), and of therapy-resistant rejection with Fas ligand gene expression (Nickel *et al.* 2001). Moreover, lower expression of regulatory transcript *FOXP3* and *CD20* was shown to be associated with the inferior outcome of early acute rejection (Viklicky *et al.* 2010).

With the development of microarray technology, a large-scale analysis of transcriptomic data was carried out. Using microarray analysis in diagnostic and protocol kidney allograft biopsies, Sarwal *et al.* (2003) was the first who provided evidence of molecular heterogeneity of renal allograft rejection with respect to pathogenesis, clinical course, and response to treatment.

In parallel with microarray, the development of more quantitative and sensitive RNA-sequencing technologies has led to the evaluation of differential gene expression in a much broader dynamic range than microarrays. In their proof-of-concept discovery study, Kurian *et al.* (2017) compared Affymetrix Gene Arrays and RNA-Seq using transplant biopsies from patients with stable allograft and with acute rejection. Both platforms demonstrated equivalent predictive performance. Despite the promising potential of sequencing, the studies published thus far on the use of RNA-Seq for detecting differentially expressed genes in kidney allografts are scarce and limited to small sample sizes (Dziubianau *et al.* 2013, Jeon *et al.* 2017).

Transcriptomic profiling of kidney graft biopsies provided some new information in clinically unclear cases (Halloran, Reeve *et al.* 2017, Halloran, Famulski *et al.* 2017, Reeve *et al.* 2016) and revealed transcripts associated with disease progression (Kadota *et al.* 2015, Hruba *et al.* 2015, Lefaucheur *et al.* 2018). Compared to semi-quantitative histology assessment, transcriptomic results are not only quantitative but demonstrate higher specificity (Sis *et al.* 2009, Naesens *et al.* 2011, Reeve *et al.* 2016, Halloran *et al.* 2014). The other advantage of molecular assessment is the ability to adequately assess small tissue samples (3 mm in length) independently of proportion of cortex/medulla (Madill-Thomsen *et al.*

Table 1. Molecular microscope body of evidence

Reference	No. of biopsies	Main findings
Development of PBTs		
Mueller *et al.* 2007	143, diagnostic	PBTs representing three biological processes in graft rejection (cytotoxic T-cell infiltration (QCAT), IFNG effects (GRIT1), and parenchymal deterioration (KT) increased in biopsies with rejection (TCMR, ABMR, mixed) compared to non-rejection biopsies.
Sis *et al.* 2009	173, diagnostic	Literature-based endothelium-associated transcripts (ENDATs, n=119) of which 25 were differentially expressed between C4d+ABMR and TCMR (e.g. *VWF, CAV1, RHOJ, MCAM, CDH5, SELE*).
Hidalgo *et al.* 2010	145, diagnostic	DSAST: higher expression in DSA+ biopsies compared to DSA-.
Development of classifiers		
Sellarés *et al.*, 2013	403, diagnostic	A molecular classifier for ABMR was built based on the top 30 differentially expressed genes between ABMR and other biopsies (e.g. *CDH13, CXCL11, DARC PLA1A, ROBO4, KLF4, TM4SF18, GNG11*).
Reeve *et al.*, 2013	403, diagnostic	A molecular classifier for TCMR was built based on the top 30 differentially expressed genes between TCMR and other biopsies (e.g. *CD96, SIRPB2, TNFSF8, BTLA, OR2I1P, IL21R*).
Famulski *et al.*, 2012	39, protocol, diagnostic	A molecular classifier for AKI was built based on the top 30 differentially expressed genes between pure AKI indication biopsies (n=28) and stable protocol biopsies (n=11), including *ITGB6, SERPINA3, MTND6, OLFM4, PTX3, LCN2a, VCAN*.
Einecke *et al.*, 2010	105, diagnostic	A molecular classifier for predicting graft loss was built based on the top 30 differentially expressed transcripts between grafts failed within 32 months after for-cause biopsy and non-failed grafts (e.g. *ITGB6, HAVCR1, LTF, ADAMTS1, SERPINA3, NNMT*, and *VCAN*).
Venner *et al.*, 2016	681, diagnostic	A molecular classifier for atrophy/fibrosis was built based on the top 30 differentially expressed genes between early biopsies with no fibrosis (ci≤1) and late biopsies with fibrosis (ci>1), including *ITGB6, MIR21, VCAN, NNMT,* and *CPA3*.
Einecke *et al.*, 2018	562, diagnostic	A molecular classifier used to predict hyalinosis at a given time post-transplant was built based on the top 30 differentially expressed genes between biopsies with ah=0 and ah>0 with time correction, including *APOBEC3G, HLA-G, BTN3A1, CXCL13, OR2I1P, PSMB8, ITGB6, MIR21, VCAN, NNMT, CPA3GCNT1, CD8B1, RAP2B, IL10RB,* and *CNPY3*.
Validation of MMDx		
Halloran *et al.*, 2013	300, diagnostic	In the INTERCOM study (6 centres), TCMR scores correlated with histological TCMR lesions (tubulitis and interstitial inflammation). A TCMR score >0.1 predicted TCMR diagnosis with an accuracy of 87 %, similar to the reference set (89 %).
Halloran *et al.*, 2013	300, diagnostic	In the INTERCOM study (6 centres), molecular ABMR diagnosis was predicted by histology-based diagnosis in 59 % of biopsies only. ABMR scores correlated with histological ABMR lesions and DSA. An ABMR score >0.2 predicted the diagnosis of ABMR or mixed rejection with an accuracy of 85 % (identical to the reference set and local assessment).
Halloran *et al.*, 2017	538, diagnostic	In the INTERCOMEX study (10 centres), MMDx was verified in real-time. Agreement between MMDx and histology was 77 % for TCMR, 77 % for ABMR, and 76 % for no rejection. Despite MMDx and histology results differing in 24 % of biopsy samples, clinicians disagreed more often with histology than with MMDX.
MMDx as a tool to refine histological criteria		
Halloran *et al.*, 2017	703, diagnostic	ABMR-positive MMDx scores correlated mainly with peritubular capillaritis, glomerulitis, glomerular double contours, DSA, and C4d staining, but much less with arterial fibrosis and vasculitis.
Reeve *et al.*, 2016	703, diagnostic	TCMR-positive MMDx scores correlated mainly with interstitial infiltrate and tubulitis, but less with arteritis (v-lesion).
Halloran *et al.*, 2018	238, diagnostic	ABMR was diagnosed in 45 % of i-IFTA biopsies with MMDx and in only 28 % based on histology. TCMR was diagnosed in 16 % of i-IFTA biopsies with MMDx and in only 8 % based on histology. The prominent feature of i-IFTA biopsies was molecular injury (e.g. acute kidney injury transcripts), which predicted graft loss.

PBTs: pathogenesis-based transcript sets, IFNG: interferon gamma, TCMR: T cell-mediated rejection, ABMR: antibody-mediated rejection, DSA: donor-specific antibody, DSAST: DSA-selective transcripts, AKI: acute kidney injury, MMDx: molecular microscope diagnostic system, i-IFTA: inflammation in areas of interstitial fibrosis-tubular atrophy

2017), at least regarding molecular scores for ABMR, TCMR, and injury, but the impact of sample size on variability should be further explored (Halloran *et al.* 2017).

Microarray-derived molecular scores for ABMR and TCMR have been used to re-evaluate histological and serological criteria for rejection (Halloran, Famulski *et al.* 2017, Reeve *et al.* 2016). Molecular assessment of transcripts indicative of endothelial injury (Sis *et al.* 2010) in renal allografts was first added as a potential diagnostic criterion for ABMR to the Banff classification in 2013 (Haas *et al.* 2014). Findings on changes to endothelial transcripts, which can help to identify kidneys undergoing antibody-mediated injury, led to the inclusion of C4d-negative cases among ABMR categories (Sis *et al.* 2009). Transcriptomic data can therefore significantly improve diagnosis, prognosis, and prediction in kidney transplant settings.

Clearly, transcriptomic analyses cannot distinguish the source of gene transcripts when a part of the biopsy is stored in RNAlater which dissolve the tissue structure. Composition of the sample, e.g. the presence of glomeruli or the amount of fibrotic or inflamed tissue, also ultimately impacts on gene expression. Kidney compartment-specific expression can be satisfactorily assessed using laser-capture microdissection (Cohen *et al.* 2002, Serinsöz *et al.* 2005, Blakey and Laszik 2004, Tycova *et al.* 2018). However, tissue processing during laser microdissection requires careful handling to preserve RNA integrity. Obtaining a reasonable amount of microdissected compartments to isolate RNA of sufficient quantity represents also a great challenge. Tissue for microdissection can either be snap-frozen (Tycova *et al.* 2018) or fixed and then paraffin-embedded (Fries *et al.* 2003, Schmid *et al.* 2003, Bockmeyer *et al.* 2018). However, RNA yields are much higher from cryosections (Cohen at al. 2002). According to our study, RNA obtained from 10 microdissected 10 micrometre-thick kidney glomeruli is sufficient for measuring around 10 transcripts by RT-qPCR (Tycova *et al.* 2018). Compartment-specific gene expression may help to identify the mechanisms involved in particular pathological processes (Seeger *et al.* 2018).

Molecular microscope: past, present, and future

Large-scale data generated by microarray require sophisticated statistical tools to extract biologically meaningful information. Based on the premise that transcript sets better reflect ongoing processes in kidney allograft than single transcripts, Halloran *et al.* introduced a definition for pathogenesis-based transcript sets (PBTs) (Table 1). The first graft pathology-related PBTs to be defined were based on experimental mouse kidney transplants (IFN-γ and rejection-induced transcripts – GRIT) (Famulski *et al.* 2006) and cell cultures (T- and B-cell-associated transcripts) (Hidalgo *et al.* 2012, Einecke *et al.* 2008). One study reported higher PBT scores in 143 consecutive human kidney transplant indication biopsies during rejection, notwithstanding similar ABMR and TCMR scores (Mueller *et al.* 2007). Sis *et al.* (2009) showed that expression of 119 endothelial-associated transcripts (selected based on the literature) was higher in ABMR than in TCMR, correlated with histopathological ABMR lesions, and predicted graft loss. Hidalgo *et al.* (2010) proposed a set of donor-specific antibody (DSA)-selective transcripts (DSASTs). Further studies by the Halloran group significantly expanded the list of PBTs to reflect the major biological allograft processes (https://www.ualberta.ca/medicine/institutes-centres-groups/atagc/research/gene-lists). A comparison of 75 most ABMR-selective transcripts derived from the INTERCOMEX study with DSA-, endothelial cell-associated transcripts (ENDAT-), and C1q-binding anti-HLA DSA-PBT sets showed that 17 transcripts were DSA-selective (22.6 %), 14 (18.7 %) were endothelial-associated, and 2 transcripts (*LST1* and *FCGR3A*) were complement-binding DSA-selective-associated (Fig. 1).

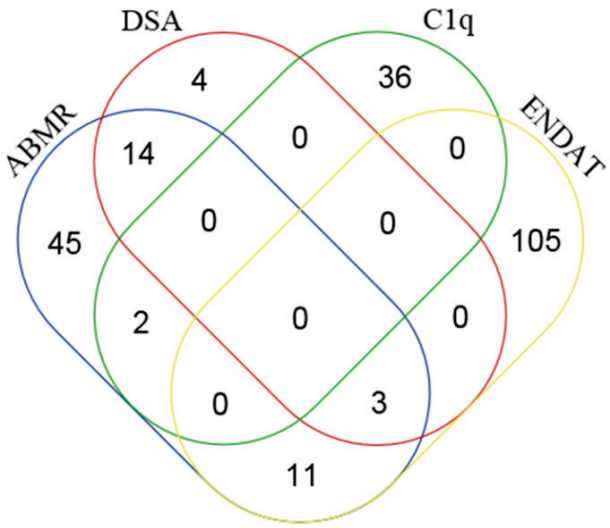

Fig. 1. The top 75 ABMR-selective transcripts derived from the INTERCOMEX study (Halloran, Reeve *et al.* 2017) were compared with particular PBTs using Venn diagrams. (http://bioinformatics.psb.ugent.be/webtools/Venn/). C1q-binding DSA PBT (Lefaucheur *et al.* 2018), DSA PBT (Hidalgo *et al.* 2010), ENDAT PBT (Sis *et al.* 2009).

The advantage and validity of the PBT concept have been further confirmed by other groups. Using the same approach, Lubetzky *et al.* (2019) compared 49 patients with (transplant glomerulopathy) TG and microvascular inflammation to 51 patients with TG but no microvascular inflammation. Transplant glomerulopathy with microvascular inflammation had increased expression of IFN-γ and rejection-induced transcripts and DSAST resembling ABMR, while TG biopsies without microvascular inflammation exhibited increased expression of cytotoxic and regulatory T cells and B cells, indicating TCMR.

Following PBTs, the Edmonton group developed several classifiers for particular kidney allograft pathologies, starting with ABMR and TCMR (Reeve *et al.* 2013, Sellares *et al.* 2013) (Table 1). Both classifiers were developed based on microarray profiling of 403 indication kidney graft biopsies. The TCMR classifier was built by comparing 35 histologically confirmed TCMR cases versus all other diagnoses, while the ABMR classifier was built by comparing both C4d-positive and C4d-negative ABMR (n=65) cases versus all other cases. Using linear discriminant analysis, the classifiers assigned an ABMR or TCMR score between 0 and 1.0 to each biopsy. The TCMR cut-off score (>0.1) resulted in 50 % sensitivity and 95 % specificity for TCMR diagnosis. The top 30 genes selected by the classifier algorithm were either primarily expressed in effector T cells and myeloid cells or were IFN-γ-inducible. The ABMR cut-off score (>0.2) resulted in 90 % specificity and 67 % sensitivity for ABMR diagnosis. The top 30 genes selected using the ABMR classifier algorithm were primarily endothelial, in addition to genes reflecting IFN-γ effects, or presence of NK cells and myeloid cells.

The diagnostic performance of these newly developed molecular classifiers for ABMR and TCMR was validated by the prospective INTERCOM study, comprising six international centres (Halloran *et al.* 2013, Halloran *et al.* 2013) working with a central diagnostic system – the Molecular Microscope Diagnostic System® (MMDx) – at Alberta Transplant Applied Genomics Centre. MMDx compares gene expression data for a given biopsy to a reference set of samples, assigning probabilistic quantitative scores to diagnose rejection, acute parenchymal injury (Famulski *et al.* 2012), atrophy/fibrosis (Venner *et al.* 2016), and non-adherence (Einecke *et al.* 2018), or to predict progression to failure (Einecke *et al.* 2010, Loupy *et al.* 2014) (Table 1). For each biopsy, a summary of molecular changes, reference values, and interpretative analysis are provided. However, classifiers were developed and validated mainly in indication biopsies performed early or late after transplantation (Reeve *et al.* 2019) and its performance in protocol biopsies to reveal subclinical rejection still needs to be validated. The feasibility of MMDx as a real-time central molecular assessment system for kidney transplant biopsy samples was further evaluated by the prospective INTERCOMEX study, which analysed 519 biopsies from 10 centres in North America and Europe. Automated reports were generated based exclusively on MMDx classifier algorithms without knowledge of histologies or HLA antibodies (Halloran, Reeve *et al.* 2017). RNA quality and yield were almost uniformly high, 96 % of biopsy samples were readable, and agreement between MMDx and histology exceeded 75 %. The authors concluded that real time central molecular assessment is feasible, the report satisfied clinicians' expectations more often than local histological assessment, and provided more weighted information for clinical decision-making.

MMDx overcomes several of the acknowledged limitations associated with microarray. The issue of insufficient reproducibility (Akalin and O'Connell 2010, Khatri *et al.* 2009, Halloran *et al.* 2014) was resolved using one microarray platform performed according to standard procedure at a single centre. Final scores were obtained using a large, fully phenotyped reference set in tandem with robust data analysis combining 12 different classifiers. Moreover, published data supported the notion that precision and accuracy of MMDx outperformed those of histology (Reeve *et al.* 2019).

Applying molecular ABMR scores permits the reassessment of histological and serological ABMR criteria (Table 1). Peritubular capillaritis, glomerulitis, glomerular double contours, DSA, and C4d staining are important features for ABMR diagnosis, whereas other Banff-recognised lesions demonstrate little (arteritis, arterial fibrous intimal thickening) or no (thrombotic microangiopathy, acute tubular injury, scarring) importance (Halloran, Famulski *et al.* 2017). Similarly, interstitial infiltrate and tubulitis are acknowledged as key histological predictors of molecular TCMR, with arteritis deemed less important (Reeve *et al.* 2016). In indication biopsies, i-IFTA is associated more with molecular injury and ABMR than with molecular TCMR (Halloran *et al.* 2018).

MMDx can complement histological criteria

provided they fall under the threshold for rejection diagnosis, or prove discordant with immunopathological or serological criteria (Table 2). However, some limitations of MMDx have been exposed. Stereotyped changes in microarray may not be entirely disease-specific. Given the small amount of tissue sampled, the heterogeneous cellular composition of the biopsy may theoretically impact on results (Akalin and O'Connell 2010). Focal changes (e.g. glomerular) tend to be evaluated less reliably than diffuse changes (Halloran *et al.* 2014, Halloran *et al.* 2015), while not all graft diseases might be evaluated accurately (e.g. glomerulonephritis, vascular and infectious pathology). It is unclear how inflammatory infiltrates in tubulointerstitial nephritis can be differentiated from acute rejection. Since MMDx is not a universal tool, non-rejection transplant pathology up to date can be better diagnosed with standard of care methods (e.g., light and fluorescent microscopy, and EM for glomerulonephritis, IHC for polyoma large T antigen, EM for viral particles and plasma PCR screen for viral load in case of suspected polyoma BK nephropathy). However, MMDx can help to exclude rejection when it is mixed with other pathology of the graft. In addition to rejection scores MMDx comprises acute kidney injury, inflammation, atrophy-fibrosis, and adherence scores that can help diagnose non-rejection transplant pathologies as well.

Prospective multicenter validation studies comparing MMDx-guided therapy with standard of care would help to strengthen the clinical significance of this new diagnostic tool and contribute to its routine adoption to clinical praxis. Moreover, in view of growing number of non-invasive molecular biomarkers for kidney graft evaluation (Suthanthiran *et al.* 2013, Roedder *et al.* 2014, Christakoudi *et al.* 2019, Huang *et al.* 2019) it will be interesting to compare diagnostic and prognostic performance of molecular microscope vs those molecular biomarkers.

Table 2. Examples of diagnostics pitfalls potentially resolved by using molecular methods

Difficult diagnoses	Histology (Banff score)	Immunohistology + serology
Active ABMR vs. no active ABMR	· MVI ≥ 1 · ATI, in the absence of any other apparent cause, MVI < 2 · Acute TMA, in the absence of any other cause, MVI < 2 · v > 0, MVI < 2	C4d-negative, DSA-positive
	· MVI ≥ 2 · v > 0, MVI < 2	C4d-negative, DSA-negative
	· MVI 0	C4d-positive, DSA ±
Chronic active ABMR vs. no chronic active ABMR	· cg > 0, MVI < 2 · Severe PTCBMML, MVI < 2 · AIF of new onset, excluding other causes, MVI < 2	C4d-negative, DSA-positive
Acute TCMR vs. no rejection	· v > 0, MVI < 2, t 0, i 0 · BL, i.e. under diagnostic threshold for rejection (t ≥ 2 and i ≥ 2)	C4d-negative, DSA-negative
Pure TCMR I or BL vs. mixed rejection	g 0, ptc ≥1, acute TCMR I or BL	C4d-negative, DSA-positive
Pure TCMR II–III vs. mixed rejection	v > 0, MVI <2	C4d-negative, DSA ±
Isolated BKVN vs. coincident with TCMR	t 2-3, i 2-3	C4d-negative, SV40-positive DSA-negative

ABMR: antibody-mediated rejection, AIF: arterial intimal fibrosis, ATI: acute tubular injury, BL: borderline lesions, BKVN: BK virus nephropathy, DSA: donor-specific antibodies, MVI: microvascular inflammation (g+ptc) score, TCMR: T cell-mediated rejection, PTCBMML: peritubular capillary basement membrane multilayering, TMA: thrombotic microangiopathy, cg: glomerular basement membrane double contour score, v: arteritis score, t: tubulitis score, i: interstitial inflammation score, g: glomerulitis score, ptc: peritubular capillaritis score, SV40: simian virus antigen 40.

Conclusions

During past several decades, molecular assessment of kidney allograft has made striking progress, from evaluation of a single gene or their sets through PBTs and molecular classifiers to MMDx. High sensitivity and specificity of molecular scores have been used to improve histological and serological criteria for graft pathology. Integrating histological, serological, and molecular assessment of renal allografts may thus provide more accurate diagnostics to improve outcome of kidney transplantation.

Conflict of Interest

There is no conflict of interest.

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