OPEN

Polyomavirus BK Nephropathy-Associated Transcriptomic Signatures: A Critical Reevaluation

Ling Pan, MD,[1] Zili Lyu, MD,[2] Benjamin Adam, MD,[3] Gang Zeng, MD, PhD,[4] Zijie Wang, MD,[4] Yuchen Huang, BS,[4] Zahidur Abedin, PhD,[5] and Parmjeet Randhawa, MD[4]

Background. Recent work using DNA microarrays has suggested that genes related to DNA replication, RNA polymerase assembly, and pathogen recognition receptors can serve as surrogate tissue biomarkers for polyomavirus BK nephropathy (BKPyVN). **Methods.** We have examined this premise by looking for differential regulation of these genes using a different technology platform (RNA-seq) and an independent set 25 biopsies covering a wide spectrum of diagnoses. **Results.** RNA-seq could discriminate T cell–mediated rejection from other common lesions seen in formalin fixed biopsy material. However, overlapping RNA-seq signatures were found among all disease processes investigated. Specifically, genes previously reported as being specific for the diagnosis of BKPyVN were found to be significantly upregulated in T cell–mediated rejection, inflamed areas of fibrosis/tubular atrophy, as well as acute tubular injury. **Conclusions.** In conclusion, the search for virus specific molecular signatures is confounded by substantial overlap in pathogenetic mechanisms between BKPyVN and nonviral forms of allograft injury. Clinical heterogeneity, overlapping exposures, and different morphologic patterns and stage of disease are a source of substantial variability in "Omics" experiments. These variables should be better controlled in future biomarker studies on BKPyVN, T cell–mediated rejection, and other forms of allograft injury, before widespread implementation of these tests in the transplant clinic.

(*Transplantation Direct* 2018;4: e339; doi: 10.1097/TXD.0000000000000752. Published online 23 January, 2018.)

Polyomavirus BK (BKV) is widespread in the general population as evidenced by a seroprevalence of up to 90%. Primary infection likely occurs via the respiratory or oral route and is followed by latency primarily in the genitourinary tract.[1] In individuals with impaired immunity, particularly kidney transplant recipients, viral reactivation leads to viruria in 30-60%, viremia in 5-30%, and BKV nephropathy (BKPyVN) in 1% to 10% of patients. The pathology of BKPyVN includes interstitial inflammation and tubulitis which can be confused with T-cell mediated rejection (TCMR). Reduction of immunosuppression after diagnosis of BKPyVN can trigger true TCMR, and further confound the histologic findings.[2] Lack of effective antiviral treatment and hesitancy to treat for rejection in the face of viral infection leads to persistent graft dysfunction. This disheartening interplay of events results in graft loss which has dropped to less than 10% in most studies conducted in the setting of viral screening and early intervention. The crux of the problem in interpreting biopsies with BKPyVN is the inability of light microscopy to quantify the relative proportions of total inflammation directed against allogeneic versus viral antigens. Glomerulitis, arteritis, and C4d deposition in peritubular capillaries can be indicative of significant rejection, but most biopsies in patients with active BKV replication do not show these findings. Thus, there is a need for additional tools to interpret the biologic

and clinical significance of inflammation that occurs in the setting of BKPyVN.[3]

Molecular approaches have elucidated the genes upregulated in virus susceptible cells after BKV infection.[4,5] However, an attempt to define whole blood gene expression profiles in patients with BK viremia primarily detected activity of the same cellular and innate immunity genes that mediate acute rejection.[6] Biopsies with BKPyVN have also been studied with the goal of characterizing a cellular milieu that might be characteristic of viral infection. In 1 study, evaluation of an extended gene panel using quantitative PCR detected an exaggerated expression of rejection-associated genes.[7] A second study that was similar in design documented upregulation of genes involved in anti-viral immune responses, but genes of potential utility in clinical differential diagnosis were not reported.[8] The most recent attempt to address this problem used the Affymetrix Human Genome U133 Plus 2.0 Array which can probe 38,500 well-characterized human genes.[9] High-throughput analysis of 10 BKPyVN biopsies revealed 209 genes with >2 fold expression compared to 30 biopsies from clinically stable patients. Four genes (LTF, CFD, RPS15, and NOSIP) were further validated using quantitative PCR in an independent set of 15 BKPyVN biopsies. Expression of these genes in BKPyVN was higher compared to biopsies obtained from patients with rejection as well as patients with stable function. However, measurements in individual patients varied over a wide range, and this suggests a need to further evaluate these promising findings across different data sets and assay platforms.

Accordingly, we have performed RNA-seq analysis to further examine the specificity of the aforementioned BKPyVN-associated genes and the generalizability of the reported conclusions.

METHODS

Clinical Material

This study was approved by the University of Pittsburgh IRB (protocol 10110393). The kidney transplant patients included in this study varied from 20 to 73 years (mean, 48.9; SD, 15.3; n = 25) with a male/female ratio of 3.7:1 (Table 1). All patients received thymoglobulin induction with a rapid 7-day corticosteroid taper. Dual-maintenance immunosuppressive therapy consisted of mycophenolate mofetil and tacrolimus. The time of biopsy varied from 9 days for patients with acute tubular injury (ATI) to 6914 days for patients with inflamed interstitial fibrosis and tubular atrophy (i-IFTA). Case selection was done from biopsies examined during routine clinical care over a 2 year period before initiation of this study. The principal author of this manuscript (P.R.) conducts a weekly biopsy conference that allows clinically validated diagnoses to be assigned to all renal allograft biopsies performed at the University of Pittsburgh. The only exclusion criterion was unavailability of sufficient residual tissue for RNA-seq. Assignment of histology diagnoses used criteria listed in the Banff 2015 Schema of renal allograft pathology.[10] Five biopsies each were selected representing stable renal function (STA), ATI, TCMR, i-IFTA of undetermined etiology, and BK polyomavirus nephropathy (BKPyVN). Biopsy

<div style="background:orange">**TABLE 1.**</div>

Clinical features of cases studied

Group	Native diagnosis	Age	Sex	PRA-I	PRA-II	TBx, d	Cr_1m	Cr_3m	Cr_6m	Cr_12m
STA	Unknown	49	M	58	37	103	2.5	2.70	3.00	2.89
	Glomerulonephritis	54	F	98	77	87	1.07	1.10	13	1.10
	Wegener's granulomatosis	76	M	0	0	105	1.35	1.35	1.50	1.56
	Hypertension	24	M	0	3	101	1.62	1.70	1.40	1.70
	Calcineurin inhibitor toxicity	60	M	0	83	90	1.60	1.40	1.50	1.30
ATI	Unknown	NA	NA	NA	NA	NA	1.61	1.53	1.53	1.86
	Lithium toxicity	40	F	0	6	361	1.20	1.10	1.00	1.00
	Hypertension	78	M	20	0	22	9.90	4.90	3.93	Dx <12 m
	Calcineurin inhibitor toxicity	49	F	0	0	9	2.30	1.60	1.2	Dx <12 m
	Hyperuricemic nephropathy	26	M	0	0	83	5.79	3.10	2.30	Dx <12 m
TCMR	Wegener's granulomatosis	24	M	0	0	95	1.60	1.60	1.6	1.62
	Hypertension	41	M	36	100	108	1.5	2.06	1.59	1.50
	Polycystic kidneys	47	F	91	0	256	0.76	0.70	0.74	0.78
	Unknown	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Diabetes mellitus	60	F	4	0	380	1.19	1.12	1	0.97
i-IFTA	Hypertension	30	M	NA	NA	2067	NA	NA	NA	NA
	Membranous nephropathy	49	F	91	97	4112	4.68	7.10	6.30	8.00
	Polycystic kidneys	58	M	0	0	96	1.10	1.30	1.40	1.20
	Chronic pyelonephritis	24	F	89	100	338	2.07	2.50	6.90	9
	Polycystic kidneys	58	F	NA	NA	6914	2.3	3.4	4.3	3.9
BKPyVn	Glomerulonephritis	54	F	98	77	366	1.1	1.2	1.2	Dx <12 m
	Diabetes mellitus	69	F	0	0	368	1.1	1.2	1.1	Dx <12 m
	Hypertension	41	M	0	0	547	7.3	9.8	NA	Dx <12 m
	Sarcoidosis	76	M	0	0	90	1.8	1.90	1.8	Dx <12 m
	Systmic lupus erythematosus	27	M	20	0	67	1.36	1.30	1.5	Dx <12 m

Cr, creatinine; Dx, diagnosis time; PRA-I, class I panel-reactive antibodies; PRA-II, class II panel-reactive antibodies; TBx, time to biopsy.
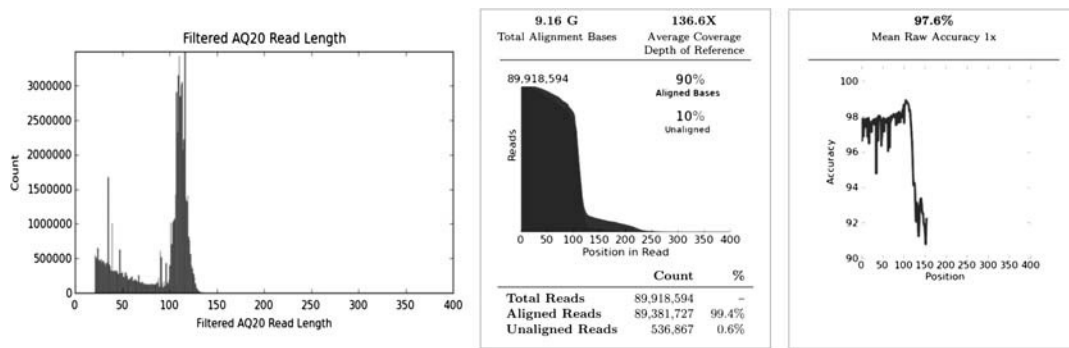
FIGURE 1. Quality control data from a representative sequencing chip. After filtering for bases with a quality value of AQ20 the most frequent sequences had a read length of 100 to 125 nucleotides (left panel). Alignment of Reads to hg19 Ampliseq Transcriptome ERCC v1 showed 90% base alignment with an average depth of coverage of 136.6×, and mean raw accuracy of 97.6% (middle and right panels).

designated as normal were protocol biopsies from stable patients. In the TCMR category, 1 biopsy was biopsy was graded as Banff 1A and 4 biopsies as Banff 1B. Biopsies labeled as IFTA had grade 2 or 3 fibrosis, and all had superimposed inflammation in nonatrophic areas, which was graded as i3 in 4 biopsies and i1 in 1 biopsy. BKPyVN was staged using American Society of Transplantation 2013 Guidelines as stage B1 (1 biopsy), stage B2 (1 biopsy), stage B3 (2 biopsies), or stage C (1 biopsy).[1] A concise summary of the clinical parameters corresponding to the biopsies studies is presented in Table 1. All patients received thymoglobulin (n = 22) or simulect (n = 3) induction with a rapid 7-day corticosteroid taper. Some patients had high PRA at time of posttransplant but no donor specific antibodies were detectable. Dual-maintenance immunosuppressive therapy consisted of mycophenolate mofetil and tacrolimus. Patients with BKV nephropathy were treated only with reduction of immunosuppression. No antiviral treatment was given. Urine viral load at biopsy varied between 3.0E + 07 and 8.84E + 10 copies/mL, whereas plasma viral load ranged from 1.43E + 03 to 9.54E + 05 copies/ml. Two patients with ATI and one with i-IFTA had low level viruria (1.43E + 03-9.54E + 05 copies/ml).

RNA-seq Protocol

Ampliseq Transcriptome analysis was performed by PrimBio Research Institute LLC (Exton, PA), using an Ion Proton sequencer Ion Proton P1 chips, IonXpress barcodes, and Torrrent_Suite 5.0.4 software. cDNA libraries were constructed from 100-ng total RNA obtained using the Ion Ampliseq Transcriptome Human Gene Expression Kit from Life Technologies (cat no. A26325) and the manufacturer's recommended protocol. The purified cDNA libraries were amplified by PCR using Library Amp Primers, and run on Agilent 2100 Bioanalyzer to determine the yield and size distribution of each library. Approximately 100 pM of pooled barcoded libraries were used for templating using Life Technologies Ion Chef 200 kit (cat no. 4488377).

Alignment and Data Analysis

Raw sequence files (fastq) were aligned to the human transcriptome (hg19) reference sequences by the StrandNGS software using default parameters. The gene and transcript annotations used were retrieved from the Ensembl data base. Aligned SAM files were used for further analysis. Quality control was assessed by the Strand NGS program, which

---

TABLE 2.

Selected genes that can discriminate TCMR from STA[a]

Symbol	Entrez gene name	Location	Type(s)
ADAMDEC1	ADAM like decysin 1	Extracellular space	Peptidase
BTLA	B and T lymphocyte-associated	Plasma membrane	Other
CD28	CD28 molecule	Plasma membrane	Transmembrane receptor
CD8A	CD8a molecule	Plasma membrane	Other
CXCL13[b]	C-X-C motif chemokine ligand 13	Extracellular space	Cytokine
IL21R	Interleukin 21 receptor	Plasma membrane	Transmembrane receptor
PLA2G2D	Phospholipase A2 group IID	Extracellular space	Enzyme
PTPN7	Protein tyrosine phosphatase, nonreceptor type 7	Cytoplasm	Phosphatase
SCML4	Sex comb on midleg-like 4 (*Drosophila*)	Nucleus	Other
SH2D1A	SH2 domain containing 1A	Cytoplasm	Other
SLA	Src-like adaptor	Plasma membrane	Other
SLAMF8[c]	SLAM family member 8	Extracellular space	Other
THEMIS	Thymocyte selection-associated	Cytoplasm	Other
TIGIT	T-cell immunoreceptor with Ig and ITIM domains	Plasma membrane	Other
TNFSF8	Tumor necrosis factor superfamily member 8	Plasma membrane	Cytokine

[a] The 15 genes listed are here are shared with a list of 29 genes used to develop a DNA microarray based classifier for TCMR.[31]

[b] Shared with Tables 3 and 4 consistent with presence of TCMR in some cases of IFTA.
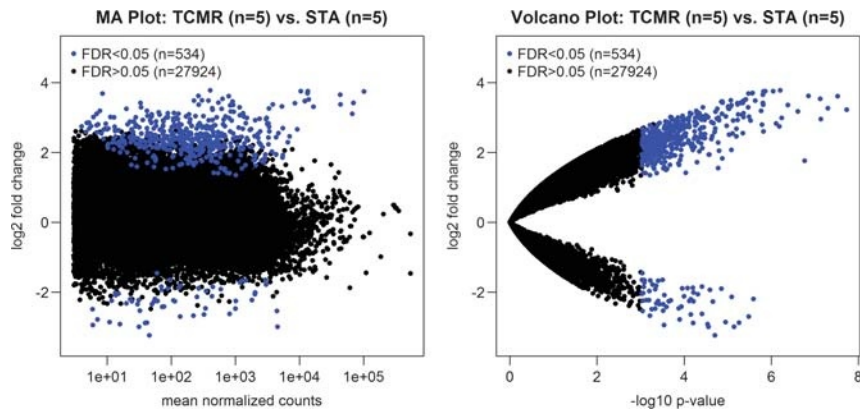
[c] Shared with Table 4.

FIGURE 2. Log ratio (M) versus mean average (A) plot for RNA-seq data derived from 5 biopsies each from the TCMR and STA groups (left panel). The same data is also illustrated in the form of a volcano plot (right panel). Genes differentially expressed at an FDR less than 0.05 are coded blue. FDR, false discovery rate.

determined the prealignment and postalignment quality of the reads for each sample. The aligned reads were then filtered based on read quality (≥15), alignment score (≥90), match count (≤1), mapping quality (≥25) and reads that failed vendors QC were removed. After filtering, the aligned reads were normalized and quantified using the DEseq algorithm by the StrandNGS program.

Statistical Analysis

The Audic Calverie Test with Benjamini-Hochberg multiple correction was used to determine significant differentially expressed genes (DEGs) based on 5 biologic replicates of each condition. After DEGs were identified, genes that had a significant fold change of 2.0× or higher compared to stable patients or other reference genes of interest were listed. Graphical representation and statistical analysis were done in R, an open source programming and software environment

supported by the R Foundation for Statistical Computing (https://en.wikipedia.org/wiki/R). Finally, pathway analysis was performed using StrandNGS software, the WikiPathways database, and ingenuity pathway analysis (IPA) on those DEGs that significantly were upregulated for each condition.

Assembly of Previously Published Genes

A list of genes regulated in allograft tissue was prepared with reference to the published literature.[6-9,11–47] Missing gene symbols, gene descriptions, and protein names were searched using the human taxonomy version of Uniprot database (www.uniprot.org/) and an Affymetrix Probeset database (http://www.affymetrix.com/analysis/netaffx/batch_query.affx?netaffx=netaffx4_annot). Transcript sets were compared with each other using IPA tools or the Microsoft Excel V-Look up function.

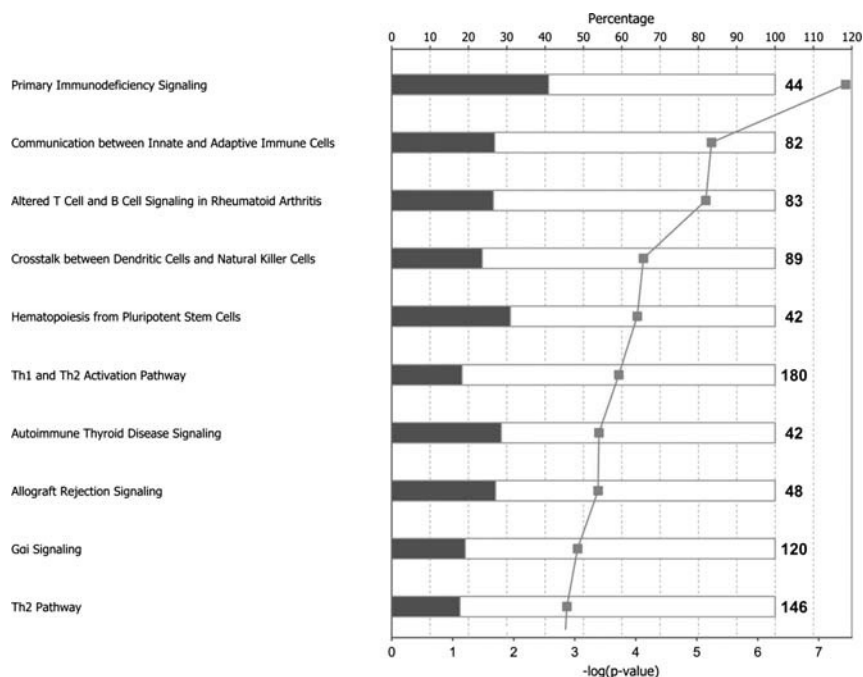**FIGURE 3.** IPA showing the top 10 biologic pathways corresponding to genes that were found to be significantly (-log p > 1.31) up-regulated in biopsies with TCMR versus STA. The largest proportion of upregulated genes (18/44, 40.9%) is seen in the "Primary immunodeficiency signaling" pathway, whereas the next largest proportion (22/82, 26.8%) belongs to the "Communication between innate and adaptive immune cells" pathway.

TABLE 3.

BKPyVN-specific genes upregulated in TCMR[a]

Symbol	Entrez gene name	Observed fold change	Published fold change	Observed, P	Published, P	Location	Type
ANKRD19P	Ankyrin repeat domain 19, pseudogene	2.6	2.1	<1E-45	4.60E-04	Other	Other
C3	Complement component 3	3.5	4.0	<1E-45	2.80E-04	Extracellular space	Peptidase
CCL18	C-C motif chemokine ligand 18	6.0	2.0	<1E-45	5.04E-03	Extracellular space	Cytokine
CD48	CD48 molecule	4.1	2.7	<1E-45	3.13E-04	Plasma membrane	Other
CXCL6	C-X-C motif chemokine ligand 6	8.4	2.2	<1E-45	7.39E-03	Extracellular space	Cytokine
CXCL13[b]	C-X-C motif chemokine ligand 13	7.3	4.9	<1E-45	2.74E-05	Extracellular space	Cytokine
IL7R	Interleukin 7 receptor	2.8	2.7	<1E-45	2.75E-04	Plasma membrane	Transmembrane receptor
ISG20	Interferon-stimulated exonuclease gene 20	3.9	2.7	<1E-45	8.76E-06	Nucleus	Enzyme
NCKAP1L	NCK-associated protein 1 like	2.9	2.1	<1E-45	2.26E-04	Plasma membrane	Other
NKAIN4[c]	Na+/K+ transporting ATPase interacting 4	4.4	3.8	<1E-45	4.83E-05	Other	Other
NKG7	Natural killer cell granule protein 7	2.2	2.9	<1E-45	5.84E-05	Plasma membrane	Other
PTPRC	Protein tyrosine phosphatase, receptor type C	2.3	2.8	<1E-45	3.07E-04	Plasma membrane	Phosphatase
REG1A	Regenerating family member 1 alpha	5.6	2.4	<1E-45	4.83E-05	Extracellular space	Growth factor
SLC1A3	Solute carrier family 1 member 3	2.3	2.1	<1E-45	8.39E-04	Plasma membrane	Transporter
BIRC3	Baculoviral IAP repeat containing 3	2.7	2.2	6.18E-14	5.13E-04	Cytoplasm	Enzyme
ITGAL[c]	Integrin subunit alpha L	2.1	2.0	6.18E-14	5.82E-03	Plasma membrane	Transmembrane receptor
APOC1[c]	Apolipoprotein C1	2.3	4.1	5.13E-12	3.55E-04	Extracellular space	Transporter
CSTA	Cystatin A	2.8	3.8	4.47E-10	2.94E-05	Cytoplasm	Other
PTTG1	Pituitary tumor-transforming 1	2.5	2.2	7.74E-09	3.12E-04	Nucleus	Transcription regulator
HTR6[c]	5-Hydroxytryptamine receptor 6	8.2	2.0	2.45E-08	2.43E-05	Plasma membrane	G-protein coupled receptor
C1orf162	Chromosome 1 open reading frame 162	2.9	2.7	5.08E-06	3.93E-04	Other	Transporter
LENEP	Lens epithelial protein	2.3	2.5	6.30E-06	2.94E-05	Cytoplasm	Other
CSPG5	Chondroitin sulfate proteoglycan 5	4.3	2.4	7.14E-04	5.02E-06	Extracellular space	Growth factor
HPR	Haptoglobin-related protein	2.3	2.1	8.30E-03	5.25E-05	Extracellular space	Peptidase
SAA1	Serum amyloid A1	2.5	3.1	3.60E-03	4.06E-06	Extracellular space	Transporter
HCP5	HLA complex P5 (nonprotein-coding)	2.7	2.5	4.30E-02	2.52E-05	Other	Other
LST1	Leukocyte-specific transcript 1	2.5	2.4	1.20E-02	9.78E-04	Plasma membrane	Other

[a] 21 genes (ANKRD19P, C3, CCL18, CD48, CXCL6, CXCL13, IL7R, ISG20, NCKAP1L, NKAIN4, NKG7, REG1A, SLC1A3, BIRC3, APOC1, HTR6, C1ORF162, LENEP, SAA1, HCP5, LST1) are shared with Table 4.

[b] Shared with Table 2 reflecting pathogenetic mechanisms in common with T cell mediated rejection.

[c] Shared with Table 5 reflecting significant tubular injury in both viral nephropathy and acute tubular necrosis.

TABLE 4.
BKPyVN-specific genes upregulated in IFTA[a]

Symbol	Entrez gene name	Observed fold change	Published fold change	Observed, P	Published, P	Location	Type
ANKRD19P	Ankyrin repeat domain 19, pseudogene	4.6	2.1	<1E-45	4.06E-04	Other	Other
BIRC3	Baculoviral IAP repeat containing 3	2.4	2.2	<1E-45	5.13E-04	Cytoplasm	Enzyme
C11ORF83	Ubiquinol-cytochrome reductase complex assembly factor 3	2.8	2.2	<1E-45	8.00E-03	Extracellular space	Other
C1QC	Complement C1q C chain	2.1	2.9	<1E-45	3.45E-05	Extracellular space	Peptidase
C3	complement component 3	5.0	4.0	<1E-45	2.80E-04	Extracellular space	Peptidase
CCL18	C-C motif chemokine ligand 18	5.4	2.0	<1E-45	5.04E-03	Extracellular space	Cytokine
CCL19	C-C motif chemokine ligand 19	2.1	4.7	<1E-45	2.18E-04	Extracellular space	Cytokine
CD14	CD14 molecule	3.1	2.0	<1E-45	7.37E-04	Plasma membrane	Transmembrane receptor
CD2	CD2 molecule	2.6	2.2	<1E-45	6.20E-04	Plasma membrane	Transmembrane receptor
CDH6	Cadherin 6	2.8	2.0	<1E-45	6.75E-04	Plasma membrane	Other
IL7R	Interleukin 7 receptor	2.4	2.7	<1E-45	2.75E-04	Plasma membrane	Transmembrane receptor
ISG20	Interferon-stimulated exonuclease gene 20	5.1	2.7	<1E-45	8.76E-06	Nucleus	Enzyme
LSP1	Lymphocyte-specific protein 1	2.1	2.0	<1E-45	1.37E-03	Cytoplasm	Other
LTF	Lactotransferrin	12.0	4.7	<1E-45	5.69E-04	Extracellular space	Peptidase
MMP7	Matrix metallopeptidase 7	4.4	2.8	<1E-45	1.24E-03	Extracellular space	Peptidase
NKAIN4[c]	Na+/K+ transporting ATPase interacting 4	5.9	3.8	<1E-45	4.83E-05	Other	Other
NNMT	Nicotinamide N-methyltransferase	2.5	4.3	<1E-45	5.84E-05	Cytoplasm	Enzyme
RAC2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	2.1	2.9	<1E-45	1.37E-03	Cytoplasm	Enzyme
REG1A	Regenerating family member 1 alpha	26.4	2.4	<1E-45	4.83E-05	Extracellular space	Growth factor
SF3B5	Splicing factor 3b subunit 5	2.7	2.1	<1E-45	4.81E-04	Nucleus	Other
NKG7	Natural killer cell granule protein 7	2.2	2.9	<1E-45	5.84E-05	Plasma membrane	Other
CFD	Complement factor D	2.1	3.0	<1E-45	2.85E-03	Extracellular space	Peptidase
NCKAP1L	NCK-associated protein 1 like	2.7	2.1	<1E-45	2.26E-04	Plasma membrane	Other
SLAMF8[b]	SLAM family member 8	2.1	2.6	<1E-45	9.94E-05	Extracellular space	Other
CD48	CD48 molecule	3.8	2.7	<1E-45	3.13E-04	Plasma membrane	Other
CD3D	CD3d molecule	2.0	3.3	<1E-45	2.89E-04	Plasma membrane	Transmembrane receptor
LTB	Lymphotoxin beta	2.8	2.7	<1E-45	4.01E-05	Extracellular space	Cytokine
GPR183[c]	G protein-coupled receptor 183	3.1	2.1	<1E-45	1.18E-04	Plasma membrane	G protein-coupled receptor
SLC1A3	Solute carrier family 1 member 3	2.6	2.1	<1E-45	8.39E-04	Plasma membrane	Transporter
RARRES1	Retinoic acid receptor responder 1	2.5	2.0	<1E-45	3.01E-03	Plasma membrane	Other
CXCL6	C-X-C motif chemokine ligand 6	27.4	2.2	<1E-45	7.39E-03	Extracellular space	Cytokine

Gene	Name	Fold	p-value	p-value	Localization	Category
PTTG1	Pituitary tumor-transforming 1	3.6	1.27E-11	3.12E-04	Nucleus	Transcription regulator
APOC1	Apolipoprotein C1	2.2	2.42E-09	3.55E-04	Extracellular space	Transporter
CXCL13[b]	C-X-C motif chemokine ligand 13	7.9	4.73E-09	2.74E-05	Extracellular space	Cytokine
LENEP	Lens epithelial protein	4.0	1.50E-08	2.94E-05	Cytoplasm	Other
HTR6[c]	5-Hydroxytryptamine receptor 6	10.9	1.77E-07	2.43E-05	Plasma membrane	G protein-coupled receptor
CSPG5	Chondroitin sulfate proteoglycan 5	7.0	4.54E-05	5.02E-06	Extracellular space	Growth factor
C1orf162	Chromosome 1 open reading frame 162	2.6	1.12E-03	3.93E-04	Other	Transporter
HCP5[c]	HLA complex P5 (nonprotein-coding)	6.4	1.45E-03	2.52E-05	Other	Other
SAA1	Serum amyloid A1	3.1	2.17E-03	4.06E-06	Extracellular space	Transporter
LST1	Leukocyte-specific transcript 1	3.2	3.02E-02	9.78E-04	Plasma membrane	Other

[a] 21 genes (ANKRD19P, C3, CCL18, CD48, CXCL6, CXCL13, IL7R, ISG20, NCKAP1L, NKAIN4, NKG7, REG1A, SLC1A3, BIRC3, APOC1, HTR6, C1ORF162, LENEP, SAA1, HCP5, LST1) are shared between Table 3 and 4 and illustrating that many cases of IFTA have underlying TCMR.
[b] Shared with Table 2 reflecting common mechanisms of tissue injury in TCMR and BKPyVN.
[c] Shared with Table 5 reflecting tubular damage in both BKPyVN and IFTA biopsies.

RESULTS

Formalin-fixed paraffin embedded (FFPE) biopsies are suitable for RNA-seq analysis: Total RNA extracted from the biopsies had an A260/280 ratio of 1.96–2.03. The RNA integrity numbers were in the range of 1.7 to 1.9 as measured by the Agilent D1000 Screen Tape Assay. These are substantially lower than that observed with frozen tissue where a typical RNA value is 7.0. However, RNA fragments greater than 200 nucleotides constituted greater than 30% of the total RNA concentration in all specimens, thus satisfying the DV200 > 30% criterion for FFPE specimen adequacy suggested by Illumina Inc. The mean sequence length in this RNA-seq data set ranged from 99 to 111 nucleotides (Figure 1). After applying filters for low quality reads, primer dimers, and polyclonal ion sphere particles, % usable reads in 10 different Ion PI Chips varied from 50-76%. This reflects the suboptimal quality of RNA in FFPE tissue. Despite this, we were able to obtain 19 to 28 million reads per sample with a quality score > Q30 (corresponding to an error rate of 0.001). Greater than 98.5% of the reads aligned to the human transcriptome with accuracy rates greater than 97.6%.

Biopsies in different diagnostic categories show overlapping RNA-seq signatures: Thirty genes upregulated in TCMR biopsies, 24 in i-IFTA biopsies, and 38 in ATI biopsies have been previously reported in microarray based studies of TCMR.[30,31,48] This confirms that FFPE tissues can give diagnostically meaningful RNA-seq signatures after low-quality sequences have been discarded by appropriate filters. The observation that i-IFTA biopsies showed upregulation of TCMR genes indicates that i-IFTA can be a manifestation of chronic T cell–mediated rejection. Gene expression overlap in biopsies with ATI and TCMR reflects the common pathogenetic mechanisms of tubular injury, irrespective of whether such injury is of immunologic or ischemic etiology. Other instances of gene lists common to biopsies with more than 1 diagnosis were also found. Thus, 32 genes upregulated in TCMR biopsies (Table 2, Figures 2 and 3), and 33 in i-IFTA biopsies were originally described in biopsies with ATI[25,49,50] (data not shown). Likewise, 433 genes upregulated in TCMR biopsies, and 396 in ATI biopsies are associated with inflammation and fibrosis in the allograft kidney.[36,51–53]

Reevaluation of genes reported to be "BKPyVN specific": A recently published high throughput analysis of 10 BKPyVN biopsies revealed 209 genes with greater than twofold expression compared with 30 biopsies from clinically stable patients.[9] To more critically evaluate the potential diagnostic value of these genes we examined their expression in our data set. Twenty-seven of these "BKPyVN-specific" genes were upregulated among the 14 362 that were differentially expressed in TCMR biopsies compared to controls (Table 3). Forty-one "BKPyVN-specific" genes (Table 4) were found to be upregulated in biopsies with i-IFTA wherein 11 198 genes were differentially expressed with respect to controls. Likewise, 16 064 genes were found to be upregulated in biopsies with ATI, and 12 of these genes were "BKPyVN specific" (Table 5). For each disease category more than half the upregulated genes coded for proteins known to localize to the plasma membrane and extracellular space. This emphasizes the role of receptor-mediated signaling, membrane-bound enzymes, growth factor, and cytokine secretion in the pathogenesis of BKPyVN. The

TABLE 5.

BKPyVN-specific genes upregulated in ATI

Symbol	Entrez gene name	Observed fold change	Published fold change	Observed, *P*	Published, *P*	Location	Type(s)
APOC1[a]	Apolipoprotein C1	6.7	4.1	<1E-45	3.55E-04	Extracellular space	Transporter
HOPX	HOP homeobox	2.5	2.9	<1E-45	4.41E-04	Nucleus	Transcription regulator
LYG1	Lysozyme g1	9.6	2.2	<1E-45	5.97E-03	Extracellular space	Other
NCAPH2	Non-SMC condensin II complex subunit H2	3.2	2.1	<1E-45	4.25E-04	Nucleus	Other
NKAIN4[b]	Na+/K+ transporting ATPase interacting 4	6.7	3.8	<1E-45	4.83E-05	Other	Other
GPR183[c]	G protein-coupled receptor 183	2.2	2.1	1.81E-11	1.18E-04	Plasma membrane	G protein-coupled receptor
FGFR1	Fibroblast growth factor receptor 1	2.9	3.5	1.92E-08	8.76E-06	Plasma membrane	Kinase
ITGAL[a]	Integrin subunit alpha L	2.1	2.0	2.89E-08	5.82E-03	Plasma membrane	Transmembrane receptor
HCP5[c]	HLA complex P5 (nonprotein-coding)	6.9	2.5	2.07E-05	2.52E-05	Other	Other
HTR6[b]	5-Hydroxytryptamine receptor 6	4.0	2.0	1.48E-04	2.43E-05	Plasma membrane	G protein-coupled receptor
WNK2	WNK lysine deficient protein kinase 2	2.3	2.0	7.16E-03	1.39E-03	Cytoplasm	Kinase
C9orf16	Chromosome 9 open reading frame 16	4.1	2.1	8.97E-03	2.13E-03	Other	Other

[a] Shared with Table 3.

[b] Shared with Table 3 and 4.

[c] Shared with Table 4.

These shared genes reflect tubular damage common to all 3 disease processes.

fold changes observed in our RNA-seq data are also quite comparable to published microarray data.

Several BKPyVN-specific genes were associated with more than 1 disease category: specifically, 5 genes were common to both ATI and TCMR, and 23 common to TCMR and IFTA. The data from our biopsy set was then examined with a specific focus on 4 genes (LTF, CFD, RPS15, and NOSIP) that were initially discovered by microarray analysis and subsequently said to be further validated using quantitative PCR as well as immunohistochemistry.[9] There was considerable overlap of gene expression across the different diagnostic categories examined (Figure 4). By DEseq analysis, LTF was found to be differentially upregulated if BKPyVN-specific genes were defined by using stable patients or TCMR patients as the reference set, but not if biopsies with ATI or i-IFTA were used as comparators. CFD, RPS15, and NOSIP were not upregulated in any of the analyses.

DISCUSSION

The primary aim of this study was to evaluate the specificity of genes reported to be selectively expressed in renal allograft biopsies with BKPyVN.[9] mRNA for several genes was found to be altered with comparable fold change in biopsies with other histologic diagnoses. The reason(s) for the discrepancy are not clear. Consideration was given to technical reasons, differences in bioinformatics analyses, and inability to control for relevant clinical as well as histologic parameters.

With respect to technology, the reported demonstration of BKPyVN-specific genes relied on analysis of fresh tissue using Affymetrix Gene Chip U133 Plus 2.0 Arrays. This array can detect transcripts derived from approximately 38 500 genes but sequence clusters needed for designing the chip were created from older databases, such as UniGene database (Build 133, 4/20/2001) and the University of California, Santa Cruz Golden-Path human genome database, April 2001 release (http://media.affymetrix.com/support/technical/datasheets/human_datasheet.pdf). In contrast, our results were obtained using RNAseq on FFPE tissues. FFPE tissue typically yields lower quality RNA but this would not explain the detection of additional genes not detected by microarrays. However, it could be argued that microarray chips can get saturated
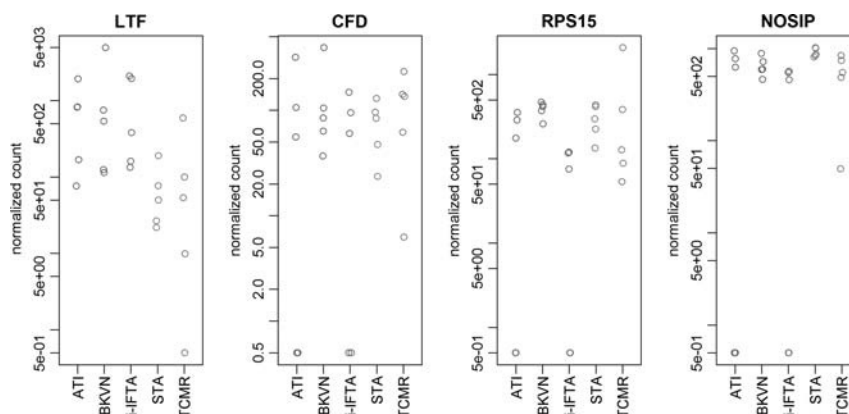

FIGURE 4. Expression of 4 previously reported 'BKPyVN specific' genes (LTF, CFD, RPS15 and NOSIP) in 5 biopsies each with ATI, BKPyVN, STA, and TCMR. Data are expressed as RNA-seq counts for the individual samples (circles) after normalization for library counts to correct for differences in library size.

by more abundant mRNAs which could result in failure to detect rare sequences. Another potential factor favoring detection by RNAseq is the initial amplification step in the Ion AmpliSeq Transcriptome Human Gene Expression Kit. This kit contains primers designed to measure the expression levels of over 20 000 human RefSeq genes over a broader dynamic range than is possible with microarray chips. Microarray and RNA-seq technology use different bioinformatics strategies to process raw data. Affymetrix chips incorporate 100 normalization control probe sets representing human housekeeping genes. This facilitates the normalization and scaling of array experiments that result in signals derived from in-situ hybridization. By comparison, the RNA-seq data in this study was processed by the DEseq program, which first normalized raw sequence count for library size using a size factors vector that corrects for varying degrees of RNA input from different samples. In RNA-seq analysis, the amplification strategy used for library preparation can also affect detection and quantitation of a mRNA populations.

The detection of BKPyVN-associated genes in biopsies with other morphologic diagnoses is biologically plausible. Thus, gene expression overlap in biopsies with BKPyVN, ATI, and TCMR reflects common pathogenetic mechanisms of tubular injury which may not depend on whether such injury is viral or nonviral in origin. In our opinion, studies using "Omics" techniques should put in more effort to control for histologic parameters, because these can be a source of substantial variability in the data obtained. It is intuitive that clinical heterogeneity, overlapping exposures (simultaneous presence of more than 1 disease), and pathology lesions would determine the associated transcriptomics profile in tissue samples: yet many gene expression studies simply classify biopsies by a single diagnostic label for the purposes of differential gene expression analysis, without detailed input from a collaborating pathologist. BKPyVN is a disease that has very different morphologic expressions in different patients. Biopsies showing acute tubular necrosis, acute interstitial nephritis, and advanced interstitial fibrosis are described. The relative proportions of these distinct morphologic and presumably molecular phenotypes in any data set will affect any putative disease-associated transcriptome signatures that can be derived from these tissues. In the study by Sigdel et al,[9,54] an attempt was made to match BKPyVN and control biopsies for major clinical variables such as donor/recipient age, % living donor kidneys, time posttransplant, and immunosuppression usage. However, there were insufficient numbers of BKPyVN biopsies to separately study individual disease phenotypes. Moreover, the degree of interstitial fibrosis and associated inflammation was neither well characterized nor effectively matched between the comparison groups.

The principal limitation of this study is that the changes in gene expression reported here have not been confirmed by a second technique, such as quantitative PCR. This was felt not to be indicated for the following reasons: (a) overlap of 30 TCMR, 24 i-IFTA, and 38 ATI genes with published DNA microarray data sets acts as an external validation by an independent platform; (b) the fold changes observed by us for these genes are comparable to prior studies; (c) indeed our P values are much more robust; and (d) the genes observed to be upregulated are supported by biologic plausibility. A second potential limitation is the small sample size.

However, it should be kept in mind that the primary aim of this study is not to report a new set of diagnostic or predictive genes but to show lack of specificity of those previously reported, and stress the need for the "Omics" research community to better control for histologic parameters to improve interinstitutional data reproducibility. This aim has been adequately accomplished by the samples studied.

In conclusion, this study highlights the problems faced by investigators seeking to discover BKPyVN-specific genes and proteins of potential diagnostic utility in the transplant clinics. It is unlikely that success will be achieved by studying small numbers of samples available to individual investigators. The transplant community should organize collaborative multicenter studies using large data sets that represent a very broad spectrum of transplant pathology including different histologic stages of BKPyVN. Control groups should consist of carefully annotated biopsies that have been collected in well-defined clinical settings and include samples with comparable inflammation, tubular injury, and fibrosis of nonviral etiology.

REFERENCES

1. Hirsch HH, Randhawa P. BK polyomavirus in solid organ transplantation. *Am J Transplant*. 2013;13(Suppl4):179–188.
2. Randhawa P. Incorporation of pathology and laboratory findings into management algorithms for polyomavirus nephropathy. *Am J Transplant*. 2013;13:1379–1381.
3. Solez K, Axelsen RA, Benediktsson H, et al. International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. *Kidney Int*. 1993;44:411–422.
4. Grinde B, Gayorfar M, Rinaldo CH. Impact of a polyomavirus (BKV) infection on mRNA expression in human endothelial cells. *Virus Res*. 2007;123:86–94.
5. Abend JR, Low JA, Imperiale MJ. Global effects of BKV infection on gene expression in human primary kidney epithelial cells. *Virology*. 2010;397:73–79.
6. Lubetzky M, Bao Y, O Broin P, et al. Genomics of BK viremia in kidney transplant recipients. *Transplantation*. 2014;97:451–456.
7. Mannon RB, Hoffmann SC, Kampen RL, et al. Molecular evaluation of BK polyomavirus nephropathy. *Am J Transplant*. 2005;5:2883–2893.
8. Girmanova E, Brabcova I, Klema J, et al. Molecular networks involved in the immune control of BK polyomavirus. *Clin Dev Immunol*. 2012;2012:972102.
9. Sigdel TK, Bestard O, Salomonis N, et al. Intragraft antiviral-specific gene expression as a distinctive transcriptional signature for studies in polyomavirus-associated nephropathy. *Transplantation*. 2016;100:2062–2070.
10. Loupy A, Haas M, Solez K, et al. The Banff 2015 Kidney Meeting Report: current challenges in rejection classification and prospects for adopting molecular pathology. *Am J Transplant*. 2017;17:28–41.
11. Adam B, Afzali B, Dominy KM, et al. Multiplexed color-coded probe-based gene expression assessment for clinical molecular diagnostics in formalin-fixed paraffin-embedded human renal allograft tissue. *Clin Transplant*. 2016;30:295–305.
12. Afzali B, Chapman E, Racape M, et al. Molecular assessment of microcirculation injury in formalin-fixed human cardiac allograft biopsies with antibody-mediated rejection. *Am J Transplant*. 2017;17:496–505.
13. Sarwal M, Sigdel T. A common blood gene assay predates clinical and histologic histological rejection in kidney and heart allografts. *Clin Transpl*. 2013:241–247.
14. Crespo-Leiro MG, Stypmann J, Schulz U, et al. Performance of gene-expression profiling test score variability to predict future clinical events in heart transplant recipients. *BMC Cardiovasc Disord*. 2015;15:120.
15. Crespo-Leiro MG, Stypmann J, Schulz U, et al. Clinical usefulness of gene-expression profile to rule out acute rejection after heart transplantation: CARGO II. *Eur Heart J*. 2016;37:2591–2601.
16. Yamani MH, Taylor DO, Rodriguez ER, et al. Transplant vasculopathy is associated with increased AlloMap gene expression score. *J Heart Lung Transplant*. 2007;26:403–406.

17. Flechner SM, Kurian SM, Head SR, et al. Kidney transplant rejection and tissue injury by gene profiling of biopsies and peripheral blood lymphocytes. *Am J Transplant*. 2004;4:1475–1489.

18. Levitsky J, Baker TB, Jie C, et al. Plasma protein biomarkers enhance the clinical prediction of kidney injury recovery in patients undergoing liver transplantation. *Hepatology*. 2014;60:2017–2026.

19. Levitsky J, Salomon DR, Abecassis M, et al. Clinical and plasma proteomic markers correlating with chronic kidney disease after liver transplantation. *Am J Transplant*. 2011;11:1972–1978.

20. Brouard S, Mansfield E, Braud C, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci U S A*. 2007;104:15448–15453.

21. Khatri P, Roedder S, Kimura N, et al. A common rejection module (CRM) for acute rejection across multiple organs identifies novel therapeutics for organ transplantation. *J Exp Med*. 2013;210:2205–2221.

22. Li L, Khatri P, Sigdel TK, et al. A peripheral blood diagnostic test for acute rejection in renal transplantation. *Am J Transplant*. 2012;12:2710–2718.

23. Li L, Wozniak LJ, Rodder S, et al. A common peripheral blood gene set for diagnosis of operational tolerance in pediatric and adult liver transplantation. *Am J Transplant*. 2012;12:1218–1228.

24. Sigdel TK, Bestard O, Tran TQ, et al. A computational gene expression score for predicting immune injury in renal allografts. *PLoS One*. 2015; 10:e0138133.

25. Famulski KS, de Freitas DG, Kreepala C, et al. Molecular phenotypes of acute kidney injury in kidney transplants. *J Am Soc Nephrol*. 2012;23: 948–958.

26. Mengel M, Chang J, Kayser D, et al. The molecular phenotype of 6-week protocol biopsies from human renal allografts: reflections of prior injury but not future course. *Am J Transplant*. 2011;11:708–718.

27. Einecke G, Kayser D, Vanslambrouck JM, et al. Loss of solute carriers in T cell-mediated rejection in mouse and human kidneys: an active epithelial injury-repair response. *Am J Transplant*. 2010;10:2241–2251.

28. De Freitas DG, Sellares J, Mengel M, et al. The nature of biopsies with "borderline rejection" and prospects for eliminating this category. *Am J Transplant*. 2012;12:191–201.

29. Famulski KS, Einecke G, Sis B, et al. Defining the canonical form of T-cell-mediated rejection in human kidney transplants. *Am J Transplant*. 2010; 10:810–820.

30. Hidalgo LG, Einecke G, Allanach K, et al. The transcriptome of human cytotoxic T cells: measuring the burden of CTL-associated transcripts in human kidney transplants. *Am J Transplant*. 2008;8:637–646.

31. Reeve J, Sellares J, Mengel M, et al. Molecular diagnosis of T cell-mediated rejection in human kidney transplant biopsies. *Am J Transplant*. 2013;13:645–655.

32. Sis B, Halloran PF. Endothelial transcripts uncover a previously unknown phenotype: C4d-negative antibody-mediated rejection. *Curr Opin Organ Transplant*. 2010;15:42–48.

33. Sis B, Jhangri GS, Bunnag S, et al. Endothelial gene expression in kidney transplants with alloantibody indicates antibody- mediated damage despite lack of C4d staining. *Am J Transplant*. 2009;9:2312–2323.

34. Sis B, Jhangri GS, Riopel J, et al. A new diagnostic algorithm for antibody-mediated microcirculation inflammation in kidney transplants. *Am J Transplant*. 2012;12:1168–1179.

35. Hidalgo LG, Sis B, Sellares J, et al. NK cell transcripts and NK cells in kidney biopsies from patients with donor-specific antibodies: evidence for NK cell involvement in antibody-mediated rejection. *Am J Transplant*. 2010; 10:1812–1822.

36. Einecke G, Reeve J, Sis B, et al. A molecular classifier for predicting future graft loss in late kidney transplant biopsies. *J Clin Invest*. 2010;120: 1862–1872.

37. Einecke G, Sis B, Reeve J, et al. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. *Am J Transplant*. 2009; 9:2520–2531.

38. Mengel M, Reeve J, Bunnag S, et al. Molecular correlates of scarring in kidney transplants: the emergence of mast cell transcripts. *Am J Transplant*. 2009;9:169–178.

39. Dosanjh A, Robison E, Mondala T, et al. Genomic meta-analysis of growth factor and integrin pathways in chronic kidney transplant injury. *BMC Genomics*. 2013;14:275.

40. Einecke G, Fairhead T, Hidalgo LG, et al. Tubulitis and epithelial cell alterations in mouse kidney transplant rejection are independent of CD103, perforin or granzymes A/B. *Am J Transplant*. 2006;6:2109–2120.

41. Famulski KS, Kayser D, Einecke G, et al. Alternative macrophage activation-associated transcripts in T-cell-mediated rejection of mouse kidney allografts. *Am J Transplant*. 2010;10:490–497.

42. Famulski KS, Sis B, Billesberger L, et al. Interferon-gamma and donor MHC class I control alternative macrophage activation and activin expression in rejecting kidney allografts: a shift in the Th1-Th2 paradigm. *Am J Transplant*. 2008;8:547–556.

43. Hidalgo LG, Sellares J, Sis B, et al. Interpreting NK cell transcripts versus T cell transcripts in renal transplant biopsies. *Am J Transplant*. 2012;12: 1180–1191.

44. Mueller TF, Einecke G, Reeve J, et al. Microarray analysis of rejection in human kidney transplants using pathogenesis-based transcript sets. *Am J Transplant*. 2007;7:2712–2722.

45. Roedder S, Sigdel T, Salomonis N, et al. The kSORT assay to detect renal transplant patients at high risk for acute rejection: results of the multicenter AART study. *PLoS Med*. 2014;11:e1001759.

46. Spivey TL, Uccellini L, Ascierto ML, et al. Gene expression profiling in acute allograft rejection: challenging the immunologic constant of rejection hypothesis. *J Transl Med*. 2011;9:174.

47. Wang E, Worschech A, Marincola FM. The immunologic constant of rejection. *Trends Immunol*. 2008;29:256–262.

48. Reeve J, Chang J, Salazar DR, et al. Using molecular phenotyping to guide improvements in the histologic diagnosis of T cell-mediated rejection. *Am J Transplant*. 2016;16:1183–1192.

49. Kieran NE, Doran PP, Connolly SB, et al. Modification of the transcriptomic response to renal ischemia/reperfusion injury by lipoxin analog. *Kidney Int*. 2003;64:480–492.

50. Famulski KS, Reeve J, de Freitas DG, et al. Kidney transplants with progressing chronic diseases express high levels of acute kidney injury transcripts. *Am J Transplant*. 2013;13:634–644.

51. Modena BD, Milam R, Harrison F, et al. Changes in urinary microbiome populations correlate in kidney transplants with interstitial fibrosis and tubular atrophy documented in early surveillance biopsies. *Am J Transplant*. 2017;17:712–723.

52. Bunnag S, Einecke G, Reeve J, et al. Molecular correlates of renal function in kidney transplant biopsies. *J Am Soc Nephrol*. 2009;20:1149–1160.

53. Sigdel TK, Gao Y, He J, et al. Mining the human urine proteome for monitoring renal transplant injury. *Kidney Int*. 2016;89:1244–1252.

54. Bottomley MJ, Chen M, Fuggle S, et al. Application of operational tolerance signatures are limited by variability and type of immunosuppression in renal transplant recipients: A cross-sectional study. *Transplant Direct*. 2017;3:e125.