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Intragraft gene expression in native kidney BK virus nephropathy versus T cell–mediated rejection: Prospects for molecular diagnosis and risk prediction

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Novel tools are needed to improve diagnostic accuracy and risk prediction in BK virus nephropathy (BKVN). We assessed the utility of intragraft gene expression testing for these purposes. Eight hundred genes were measured in 110 archival samples, including a discovery cohort of native kidney BKVN (n = 5) vs pure T cell–mediated rejection (TCMR; n = 10). Five polyomavirus genes and seven immune-related genes (five associated with BKVN and two associated with TCMR) were significantly differentially expressed between these entities (FDR < 0.05). These three sets of genes were further evaluated in samples representing a spectrum of BK infection (n = 25), followed by a multicenter validation cohort of allograft BKVN (n = 60) vs TCMR (n = 10). Polyomavirus 5-gene set expression reliably distinguished BKVN from TCMR (validation cohort AUC = 0.992), but the immune gene sets demonstrated suboptimal diagnostic performance (AUC ≤ 0.720). Within the validation cohort, no significant

Abbreviations: AUC, area under the curve; BKVN, BK polyomavirus nephropathy; FDR, false discovery rate; FFPE, formalin-fixed, paraffin-embedded; LCM, laser capture microdissection; PVN, polyomavirus nephropathy; ROC, receiver operating characteristic; SV40, simian virus 40; TCMR, T cell–mediated rejection.

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differences in index biopsy gene expression were identified between BKVN patients demonstrating resolution (n = 35), persistent infection (n = 14) or de novo rejection (n = 11) 6 months following a standardized reduction in immunosuppression. These results suggest that, while intragraft polyomavirus gene expression may be useful as an ancillary diagnostic for BKVN, assessment for concurrent TCMR and prediction of clinical outcome may not be feasible with current molecular tools.

KEYWORDS

biopsy, clinical research/ practice, infection and infectious agents - viral: BK/ JC/ polyoma, infectious disease, kidney transplantation/ nephrology, molecular biology: mRNA/ mRNA expression, pathology/ histopathology, rejection: T cell–mediated (TCMR)

1 | INTRODUCTION

BK polyomavirus nephropathy (BKVN) remains a significant infectious complication of kidney transplantation, affecting 1%-10% of renal allografts.[1,2] Timely reduction of immunosuppression is the only effective treatment for BKVN, resulting in resolution in approximately 80% of patients, but carrying a risk of postintervention rejection in about 10%.[2]

Definitive diagnosis of BKVN requires biopsy-proven nephropathy with viral confirmation by immunohistochemistry or in situ hybridization.[1,3,4] Unfortunately, renal allograft biopsies can be falsely negative due to sampling error, suboptimal sensitivity, or early/resolving disease.[5-7] Despite requiring opposite treatments, BKVN and T cell–mediated rejection (TCMR) can exhibit overlapping clinical and histological features, presenting a diagnostic dilemma with significant implications for patient management.[1,7,8] Furthermore, a complex and currently unresolvable inter-relationship between viral reactivation, inflammation, scarring and allograft failure results in highly unpredictable clinical outcomes in patients with BKVN.[6,9,10] Novel tools are thus needed to help confirm the diagnosis and predict response to treatment.

Intragraft gene expression analysis has revolutionized the characterization of renal allograft pathology[11,12] and has the potential to facilitate improved assessment of BKVN. However, previous attempts to evaluate differences in intragraft gene expression between BKVN and TCMR, using microarrays[13,14] and RNA sequencing,[15] have been limited by an inability to definitively exclude the presence of concurrent TCMR in renal allograft biopsies with BKVN. A potential novel strategy for addressing this limitation is to analyze cases of BKVN occurring in the native kidneys of non-renal transplant patients.[16-19] Unfortunately, examples of biopsy-proven native kidney BKVN are relatively rare and difficult to prospectively collect for gene expression analysis using traditional platforms requiring fresh tissue samples. However, the novel NanoString® nCounter® gene expression system now provides the opportunity to reliably assess archival formalin-fixed paraffin-embedded (FFPE) tissue,[20,21] allowing for retrospective collection and analysis of such cases. Furthermore, this technology allows gene expression analysis to be performed on the same tissue assessed with histology, permitting direct molecular-histologic correlation and facilitating spatially resolved transcriptomics in combination with other tools such as laser capture microdissection (LCM).

The objectives of this study were to exploit the unique advantages of NanoString® technology to (1) identify the molecular differences and similarities between pure native kidney BKVN and pure TCMR, and (2) assess the utility of these gene expression signatures for clinical diagnosis and risk prediction in BKVN.

2 | MATERIALS AND METHODS

2.1 | Study samples

This study was approved by the institutional review board at the University of Alberta (Pro00034887). A total of 110 archival FFPE human samples were included, consisting of a 40-sample discovery/training cohort and a 70-sample validation/prediction cohort, as outlined in Figure 1 and detailed below.

2.2 | Discovery/training cohort

Five previously published cases of native kidney BKVN (Figure 2A), for which residual FFPE biopsy tissue was still available, were obtained from collaborators at Columbia University Medical Center, Dalhousie University, and University of British Columbia. These cases of native BKVN occurred in the setting of immunosuppression due to heart transplantation,[17] bone marrow transplantation,[18] chronic lymphocytic leukemia,[18] pulmonary tuberculosis and diabetes mellitus,[18] and liver transplantation.[19] Ten renal allograft biopsies from the same time period (2006-2015) with diagnoses of "pure TCMR" (defined as at least Banff grade I TCMR[22] without evidence of donor specific antibodies or polyomavirus infection by urine/blood PCR or histology/immunohistochemistry) were retrieved from the pathology archive at the University of Alberta Hospital (Figure 2B).

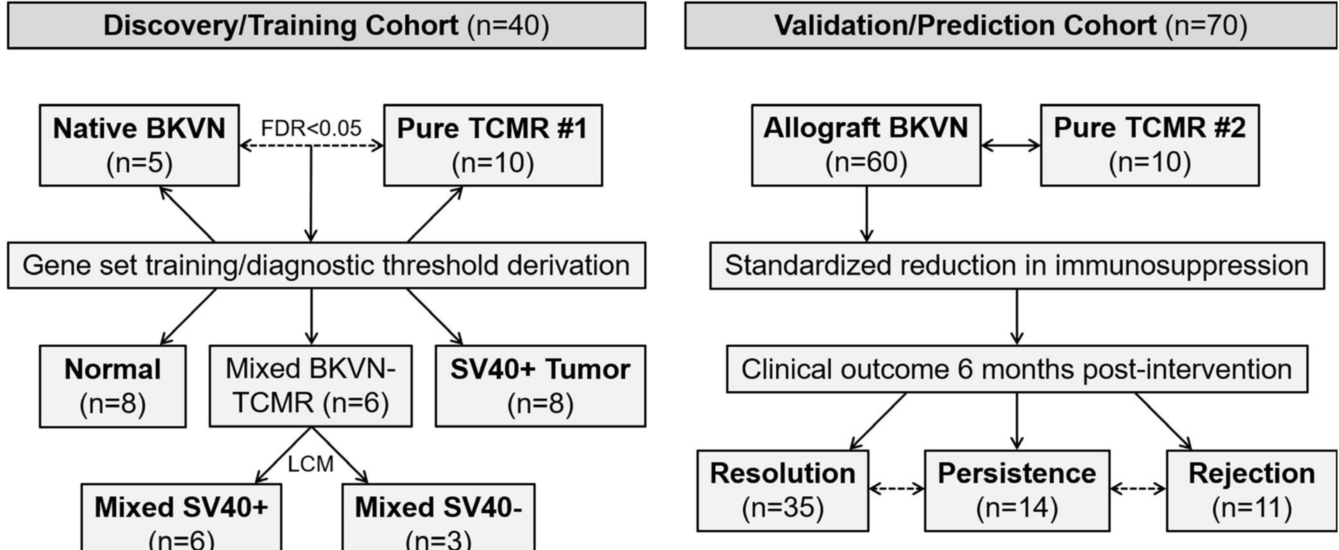

FIGURE 1 Study cohort. Dashed arrows indicate groups compared with exploratory differential gene expression analysis. BKVN, BK virus nephropathy; FDR, false discovery rate; LCM, laser capture microdissection; Mixed, mixed BKVN-TCMR; SV40, simian virus 40 immunohistochemistry; TCMR, T cell–mediated rejection

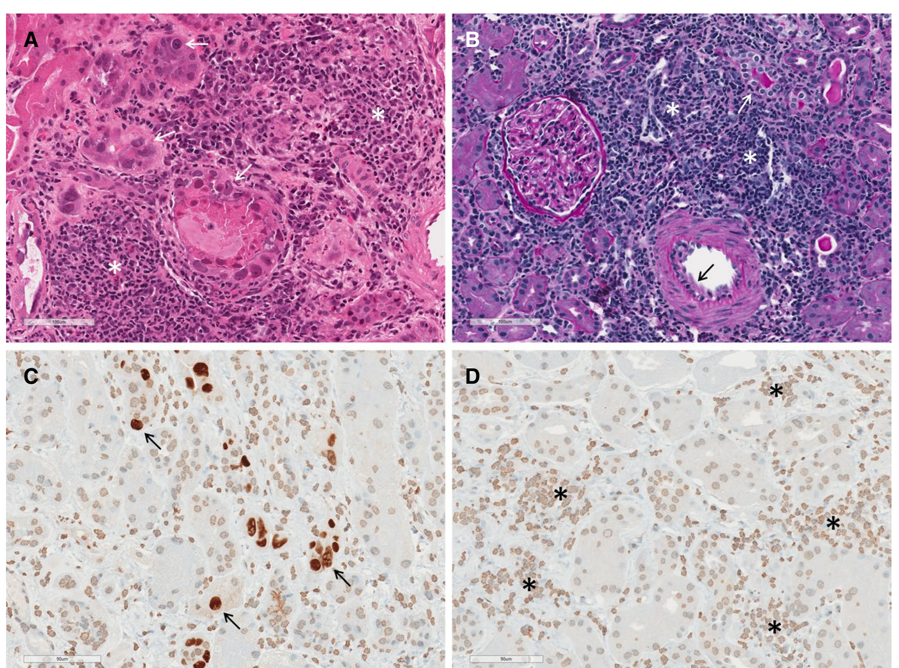

FIGURE 2 Representative photomicrographs of BKVN and TCMR. A, Native kidney BKVN with tubular viral inclusions (arrows) and background interstitial inflammation (asterisks); H&E stain. B, Pure TCMR with interstitial inflammation (asterisks), tubulitis (white arrow) and intimal arteritis (black arrow); PAS stain. C, SV40-positive area from a case of mixed BKVN-TCMR, with numerous positive viral inclusions (arrows); SV40 immunohistochemistry. D, SV40-negative area from the same case as (C), with interstitial inflammation (asterisks) but no viral inclusions; SV40 immunohistochemistry. BKVN, BK virus nephropathy; SV40, simian virus 40; TCMR, T cell–mediated rejection [Color figure can be viewed at wileyonlinelibrary.com]

Twenty-five additional samples were recruited for gene set training and diagnostic threshold determination; these included eight histologically normal, postreperfusion baseline renal allograft biopsies from the University of Alberta, eight cases of simian virus 40 (SV40) immunohistochemistry-positive urothelial carcinoma ("SV40 + Tumor"; obtained from collaborators at Hannover Medical School, University of Michigan, and University of North Carolina), and six samples representing SV40-positive areas and three samples representing SV40-negative areas from six renal allograft nephrectomies with mixed BKVN and TCMR (obtained from collaborators at Emory University, Hannover Medical School, University of British Columbia, University of Maryland, University of Pittsburgh, and Medical University of Vienna).

2.3 | Validation/prediction cohort

To validate the findings from our discovery/training cohort and evaluate the role for molecular risk prediction in BKVN, a separate cohort of renal allograft biopsies was recruited from three medical centers utilizing a standardized protocol of immunosuppression reduction for the treatment of biopsy-proven BKVN,[1] including Necker and

Saint Louis Hospitals, Paris, France, and Vienna General Hospital, Vienna, Austria. Sixty index BKVN biopsies from 2006-2017, with sufficient residual FFPE tissue and available clinical data, were obtained from these centers and categorized into one of three 6-month postintervention clinical outcome groups: (1) resolution of viremia/BKVN (n = 35; defined as serum BK viral load < 2000 copies/mL at 6 months postindex biopsy/intervention, and no biopsy-proven rejection or persistent BKVN during those 6 months); (2) persistence of viremia/BKVN (n = 14; defined as serum BK viral load > 10 000 copies/mL at 6 months postintervention, or persistent SV40-positive BKVN in follow-up biopsy but no biopsy-proven rejection); or (3) de novo rejection (n = 11; defined as any biopsy-proven rejection within 6 months of intervention). A second set of 10 biopsies with pure TCMR (using the same criteria as above) was recruited from the University of Alberta for independent diagnostic performance assessment (n = 10).

2.4 | Clinical and pathology data

For the native BKVN cases, clinical and pathology data were obtained from previously published information. For the pure TCMR and validation/prediction cohort cases, clinical data were retrieved from local medical records, and original histology slides were reviewed, scored and classified by local pathologists (BAA, FD, MR, HR) according to the 2017 Banff classification.[3,22]

2.5 | Laser capture microdissection

For the six renal allograft nephrectomies with mixed BKVN-TCMR, areas with positive SV40 immunohistochemistry staining ("Mixed SV40+," Figure 2C) and areas without SV40 staining or viral inclusions ("Mixed SV40−," Figure 2D) were identified. These areas were then isolated from three to five consecutive 20-µm sections using an ArcturusXT Laser Capture Microdissection System (Life Technologies, Burlington, ON), according to manufacturer instructions. Sufficient RNA for downstream gene expression analysis was obtained for six of the "Mixed SV40+" and three of the "Mixed SV40−" samples.

2.6 | Gene expression analysis

RNA extraction and gene expression analysis were performed as previously described.[21,23,24] Briefly, three to five consecutive 20-µm sections were obtained from each FFPE block and RNA was isolated using the RNeasy FFPE Kit (Qiagen). RNA concentration and purity were measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Gene expression was quantified using a NanoString nCounter FLEX Analysis System (NanoString Technologies, Seattle, WA), as per manufacturer recommendations. For this study, we utilized the 770-gene nCounter PanCancer Immune Profiling Panel (https://www.nanostring.com/products/gene-expression-panels/gene-expression-panels-overview/hallmarks-cancer-gene-expression-panel-collection/pancancer-immune-profiling-panel) plus 30 additional custom genes, including five polyomavirus genes (*Agnoprotein*, *LTAg*, *VP1*, *VP2*, *VP3*) and 25 additional immune-related genes previously reported to be associated with TCMR.[25] This resulted in a total of 800 genes being analyzed for each sample, including 760 experimental genes and 40 housekeeping genes. Quality control assessment and data normalization were performed using the default settings in nSolver Analysis Software Version 4.0 (NanoString Technologies).

2.7 | Statistical analysis

All post-normalization statistical analysis was performed using R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). Categorical data are presented as counts (percent) and continuous and ordinal data are presented as mean ± SD (range). Class comparison analyses were performed using Fisher's exact test (fisher.test function in stats package) for categorical data and Mann-Whitney U-test (wilcox.test function in stats package) for ordinal and continuous data. Log2 normalized transcript counts were used for individual gene analyses and mean log2 normalized counts were used for aggregate gene set analyses. Differential gene expression was assessed using linear regression (lm function in stats package) with a false discovery rate (FDR) threshold of 0.05 (p.adjust function in stats package). Receiver operating characteristic (ROC) curve analysis (roc function in pROC package) was used to assess diagnostic performance. Validation cohort performance was evaluated using diagnostic thresholds derived from the training cohort. Youden's J-statistic was utilized for defining these diagnostic thresholds.[26] Spearman's rank correlation coefficient (cor function in stats package) was used to assess correlation between genes and to characterize relationships between gene expression and histology. Death-censored renal allograft survival was assessed using Kaplan-Meier curves (ggsurvplot function in survminer package) and log-rank test (coxph function in survival package). Statistical significance was considered at $P < .05$.

3 | RESULTS

3.1 | Baseline characteristics

Clinical and histological features of the discovery and validation cohorts are presented in Table 1. There were no significant differences within and between these cohorts with regards to patient age, sex, cause of end-stage renal disease, donor status, retransplantation, maintenance immunosuppression, serum creatinine, proteinuria, presence of donor specific antibodies, graft loss, and most histology lesions ($P \geq .05$). Within the discovery cohort, no significant differences were identified between the native BKVN

TABLE 1 Clinical and histological characteristics of discovery and validation cohorts

Feature	Discovery cohort (n = 15)		Validation cohort (n = 70)		*P*-value (native BKVN vs pure TCMR #1)[b]	*P*-value (allograft BKVN vs pure TCMR #2)[b]	*P*-value (native BKVN vs allograft BKVN)[b]	*P*-value (pure TCMR #1 vs pure TCMR #2)[b]
	Native BKVN (n = 5)[a]	Pure TCMR #1 (n = 10)[a]	Allograft BKVN (n = 60)[a]	Pure TCMR #2 (n = 10)[a]				
Clinical features (at time of biopsy)								
Patient age, y	48 ± 25 (17-73)	47 ± 11 (29-65)	52 ± 15 (20-80)	47 ± 21 (4-63)	.817	.684	.374	.658
Sex (male)	5 (100)	6 (60)	36 (60)	5 (50)	.231	.731	.149	1.000
Cause of end-stage renal disease								
Diabetes	N/A	3 (30)	8 (13)	4 (40)	N/A	.060	N/A	1.000
Glomerulonephritis	N/A	2 (20)	14 (23)	3 (30)	N/A	.696	N/A	1.000
Polycystic kidney disease	N/A	2 (20)	13 (22)	1 (10)	N/A	.674	N/A	1.000
Reflux/obstructive nephropathy	N/A	1 (10)	8 (13)	0 (0)	N/A	.591	N/A	1.000
Unknown	N/A	2 (20)	17 (28)	2 (20)	N/A	.717	N/A	1.000
Deceased donor	N/A	7 (70)	49 (82)	8 (80)	N/A	1.000	N/A	1.000
Retransplant	N/A	0 (0)	11 (18)	1 (10)	N/A	1.000	N/A	1.000
Maintenance immunosuppression								
Tacrolimus-based	N/A	9 (90)	59 (98)	10 (100)	N/A	1.000	N/A	1.000
Sirolimus-based	N/A	1 (10)	0 (0)	0 (0)	N/A	1.000	N/A	1.000
Belatacept-based	N/A	0 (0)	1 (2)	0 (0)	N/A	1.000	N/A	1.000
Immunosuppression duration, mo	29 ± 31 (9-84)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Time posttransplant, mo	N/A	10 ± 10 (1-33)	16 ± 23 (1-154)	12 ± 28 (1-91)	N/A	**.011**	N/A	.251
Serum creatinine, mg/dL	3.1 ± 1.1 (1.9-4.5)	2.9 ± 1.9 (1.4-7.9)	2.2 ± 0.9 (1.1-4.8)	3.0 ± 2.4 (0.6-7.6)	.390	.815	.059	.545
Proteinuria, g/g creatinine	N/A	0.8 ± 1.2 (0.0-3.2)	0.4 ± 0.3 (0.1-1.6)	0.6 ± 0.5 (0.0-1.6)	N/A	.143	N/A	.859
Presence of BK viremia	5 (100)	0 (0)	60 (100)	0 (0)	**.0003**	**<.0001**	1.000	1.000
Presence of DSA	N/A	0 (0)	9 (15)	0 (0)	N/A	.339	N/A	1.000
Graft loss	N/A	3 (30)	11 (18)	3 (30)	N/A	.407	N/A	1.000
Time from biopsy to graft loss, mo	N/A	23 ± 5 (17-26)	32 ± 21 (2-66)	22 ± 14 (13-38)	N/A	.469	N/A	.700

(Continues)

TABLE 1 (Continued)

Feature	Discovery cohort (n = 15)		Validation cohort (n = 70)					
	Native BKVN (n = 5)[a]	Pure TCMR #1 (n = 10)[a]	Allograft BKVN (n = 60)[a]	Pure TCMR #2 (n = 10)[a]	*P*-value (native BKVN vs pure TCMR #1)[b]	*P*-value (allograft BKVN vs pure TCMR #2)[b]	*P*-value (native BKVN vs allograft BKVN)[b]	*P*-value (pure TCMR #1 vs pure TCMR #2)[b]
Biopsy features								
SV40 IHC positive	4 (80)[c]	0 (0)	60 (100)	0 (0)	**.004**	**<.0001**	.077	1.000
Tissue viral load (pvl)	2.4 ± 0.5 (2-3)	0.0 ± 0.0 (0-0)	2.1 ± 0.9 (1-3)	0.0 ± 0.0 (0-0)	**.0003**	**<.0001**	.519	1.000
Interstitial inflammation (i)	2.6 ± 0.5 (2-3)	2.4 ± 0.7 (1-3)	0.8 ± 1.1 (0-3)	2.0 ± 1.2 (0-3)	.679	**.048**	**.003**	.639
Total interstitial inflammation (ti)	N/A	2.2 ± 0.8 (1-3)	1.4 ± 1.1 (0-3)	2.5 ± 0.6 (2-3)	N/A	.050	N/A	.687
Tubulitis (t)	N/A	2.6 ± 0.5 (2-3)	1.4 ± 1.4 (0-3)	2.2 ± 0.8 (1-3)	N/A	.109	N/A	.257
Glomerulitis (g)	N/A	0.3 ± 0.7 (0-2)	0.2 ± 0.6 (0-3)	0.4 ± 0.9 (0-2)	N/A	.746	N/A	1.000
Arteritis (v)	N/A	0.6 ± 0.7 (0-2)	0.0 ± 0.0 (0-0)	1.0 ± 0.7 (0-2)	N/A	**<.0001**	N/A	.315
Peritubular capillaritis (ptc)	N/A	0.9 ± 0.9 (0-2)	0.3 ± 0.7 (0-3)	1.8 ± 1.1 (0-3)	N/A	**.001**	N/A	.120
Interstitial fibrosis (ci)	N/A	0.8 ± 1.0 (0-3)	1.4 ± 1.0 (0-3)	0.9 ± 1.0 (0-3)	N/A	.163	N/A	.776
Tubular atrophy (ct)	N/A	0.9 ± 1.0 (0-3)	1.3 ± 1.0 (0-3)	1.0 ± 0.9 (0-3)	N/A	0.358	N/A	.776
Transplant glomerulopathy (cg)	N/A	0.2 ± 0.6 (0-2)	0.0 ± 0.3 (0-2)	0.0 ± 0.0 (0-0)	N/A	.809	N/A	.572
Mesangial matrix expansion (mm)	N/A	0.5 ± 0.5 (0-1)	0.4 ± 0.8 (0-3)	0.4 ± 0.5 (0-1)	N/A	.460	N/A	.576
Arterial fibrointimal thickening (cv)	N/A	0.8 ± 0.9 (0-2)	1.6 ± 1.0 (0-3)	0.6 ± 0.9 (0-2)	N/A	**.044**	N/A	.736
Arteriolar hyalinosis (ah)	N/A	0.3 ± 0.5 (0-1)	1.0 ± 1.0 (0-3)	0.2 ± 0.4 (0-1)	N/A	**.009**	N/A	.651
C4d score	N/A	0.1 ± 0.3 (0-1)	0.4 ± 0.7 (0-2)	0.0 ± 0.0 (0-0)	N/A	.253	N/A	.572

Abbreviations: BKVN, BK virus nephropathy; N/A, not applicable/available; TCMR, T cell–mediated rejection.

[a]Data presented as count (%) for categorical data and mean ± SD (range) for continuous/ordinal data.

[b]*P*-values calculated using Mann-Whitney U-test for continuous/ordinal data and Fisher's exact test for categorical data.

[c]One native BKVN biopsy was SV40 negative but demonstrated viral inclusions on electron microscopy (ref. 17).

and pure TCMR groups, other than the definitional discrepancies between BK viremia, SV40 positivity and tissue viral load. Within the validation cohort, the allograft BKVN group (vs the pure TCMR group) had a longer duration between transplant and biopsy (16 ± 23 vs 12 ± 28 months, P = .011), less interstitial inflammation (i-score: 0.8 ± 1.1 vs 2.0 ± 1.2, P = .048), less arteritis (v-score: 0.0 ± 0.0 vs 1.0 ± 0.7, P < .0001), less peritubular capillaritis (ptc-score: 0.3 ± 0.7 vs 1.8 ± 1.1, P = .001), more arterial fibrointimal thickening (cv-score: 1.6 ± 1.0 vs 0.6 ± 0.9, P = .044), and more arteriolar hyalinosis (ah-score: 1.0 ± 1.0 vs 0.2 ± 0.4, P = .009). Compared with the allograft BKVN cohort, the native BKVN group had significantly more interstitial inflammation (i-score: 2.6 ± 0.5 vs 0.8 ± 1.1, P = .003) and a trend towards higher serum creatinine (3.1 ± 1.1 vs 2.2 ± 0.9 mg/dL, P = .059), but similar tissue viral load (pvl-score: 2.4 ± 0.5 vs 2.1 ± 0.9, P = .519). There were no significant differences between the discovery and validation cohort TCMR groups.

3.2 | RNA and quality control

Mean RNA yield was 69.0 ± 65.7 ng/µL (range: 7.4-368.2 ng/µL) with a mean A_{260}/A_{280} RNA purity ratio of 1.90 ± 0.17 (1.51-2.06). No quality control or normalization flags were encountered. The mean positive control normalization factor was 0.94 ± 0.43 (0.51-2.61; manufacturer-recommended acceptable range: 0.3-3) and mean housekeeping gene normalization factor was 1.10 ± 1.39 (0.22-8.89; acceptable range: 0.1-10).

3.3 | Discovery cohort: Gene expression in native kidney BKVN vs pure TCMR

Exploratory volcano plot analysis demonstrated 12/760 genes to have significant differential expression between native BKVN and pure TCMR (FDR < 0.05) (Figure 3A). These included all five polyomavirus genes as well as five human immune-related genes with relatively higher expression in BKVN (*CXCL6*, *FCGR2B*, *CD1C*, *MAP3K5*, *MEF2C*) and two human immune-related genes with relatively higher expression in TCMR (*VEGFA*, *ITGA6*). The magnitude of differential expression and fold change observed with the polyomavirus genes was markedly greater than that seen with the seven immune genes (Table 2).

3.4 | Training cohort: Gene set diagnostic threshold derivation

These three groups of differentially expressed genes were combined into aggregate "Polyomavirus," "BKVN-Immune," and "TCMR-Immune" gene sets by calculating the mean of their log2 normalized counts. The potential diagnostic utility of these three gene sets was evaluated in an expanded 40-sample training cohort representing a spectrum of BK virus infection (Figure 3B). The native BKVN, Mixed SV40+, Mixed SV40− and SV40+ Tumor groups demonstrated significantly higher Polyomavirus and BKVN-Immune gene set expression than pure TCMR (P = .0002-0.034), which itself exhibited higher expression than the normal controls (P = .00005-0.003). Pure TCMR displayed significantly higher TCMR-Immune gene set expression than native BKVN (P = .0007), Mixed SV40+ (P = .0002) and Mixed SV40− (P = .007), but not SV40+ Tumor (P = .203). The normal controls had even higher expression of the TCMR-Immune gene set than pure TCMR (P = .00009), indicating that relatively higher expression in pure TCMR vs the BKVN groups reflects attenuated downregulation rather than true upregulation. Native BKVN exhibited higher BKVN-Immune gene set expression than Mixed SV40+ (P = .030), but no other statistically significant differences were observed between native BKVN and Mixed SV40+/SV40− (P = .071-1.000). There were no statistically significant differences in gene set expression between Mixed SV40+ and Mixed SV40− (P = .167-0.381).

The diagnostic performance of these gene sets was assessed by grouping the training cohort cases into clinical BK-positive (native BKVN, Mixed SV40+, Mixed SV40−, SV40+ Tumor) and BK-negative (pure TCMR, Normal) categories. Class comparison and ROC curve analyses revealed excellent discrimination between these two groups with all three gene sets (Figure 4A,B). The Polyomavirus 5-gene set demonstrated the strongest diagnostic performance (AUC = 0.992), followed by the BKVN-Immune 5-gene set (AUC = 0.952) and TCMR-Immune 2-gene set (AUC = 0.934) (Table 2). The Polyomavirus 5-gene set (as well as each of its constituent genes, but not the immune gene sets) was more sensitive than histology alone for identifying BK-positive cases (AUC: 0.992 vs 0.932, accuracy: 0.975 vs 0.925, sensitivity: 0.955 vs 0.864, NPV: 0.947 vs 0.857, specificity: 1.0 vs 1.0, PPV: 1.0 vs 1.0, respectively).

3.5 | Validation cohort: Molecular diagnosis of BKVN in renal allograft biopsies

To validate these discovery/training cohort findings, gene set expression was then evaluated in a separate multicenter cohort consisting of 60 renal allograft biopsies with BKVN and 10 with pure TCMR, using diagnostic thresholds derived from the training cohort (Polyomavirus 5-gene set: 2.670, BKVN-Immune 5-gene set: 4.984, TCMR-Immune 2-gene set: 6.597). The Polyomavirus 5-gene set demonstrated similarly excellent performance in the validation cohort (AUC = 0.992), confirming its utility for the identification of BKVN (Figure 4C,D, Table 2). However, the BKVN-Immune and TCMR-Immune gene sets displayed suboptimal validation cohort performance (AUC = 0.720 and AUC = 0.633, respectively).

To further understand the diagnostic relevance of intragraft polyomavirus gene expression in the context of currently available biopsy features, correlation with Banff histology scores was assessed within the allograft BKVN validation cohort (n = 60). Polyomavirus 5-gene set expression demonstrated only moderate correlation with tissue viral load (rho = 0.443, P = .0004) and Banff polyomavirus

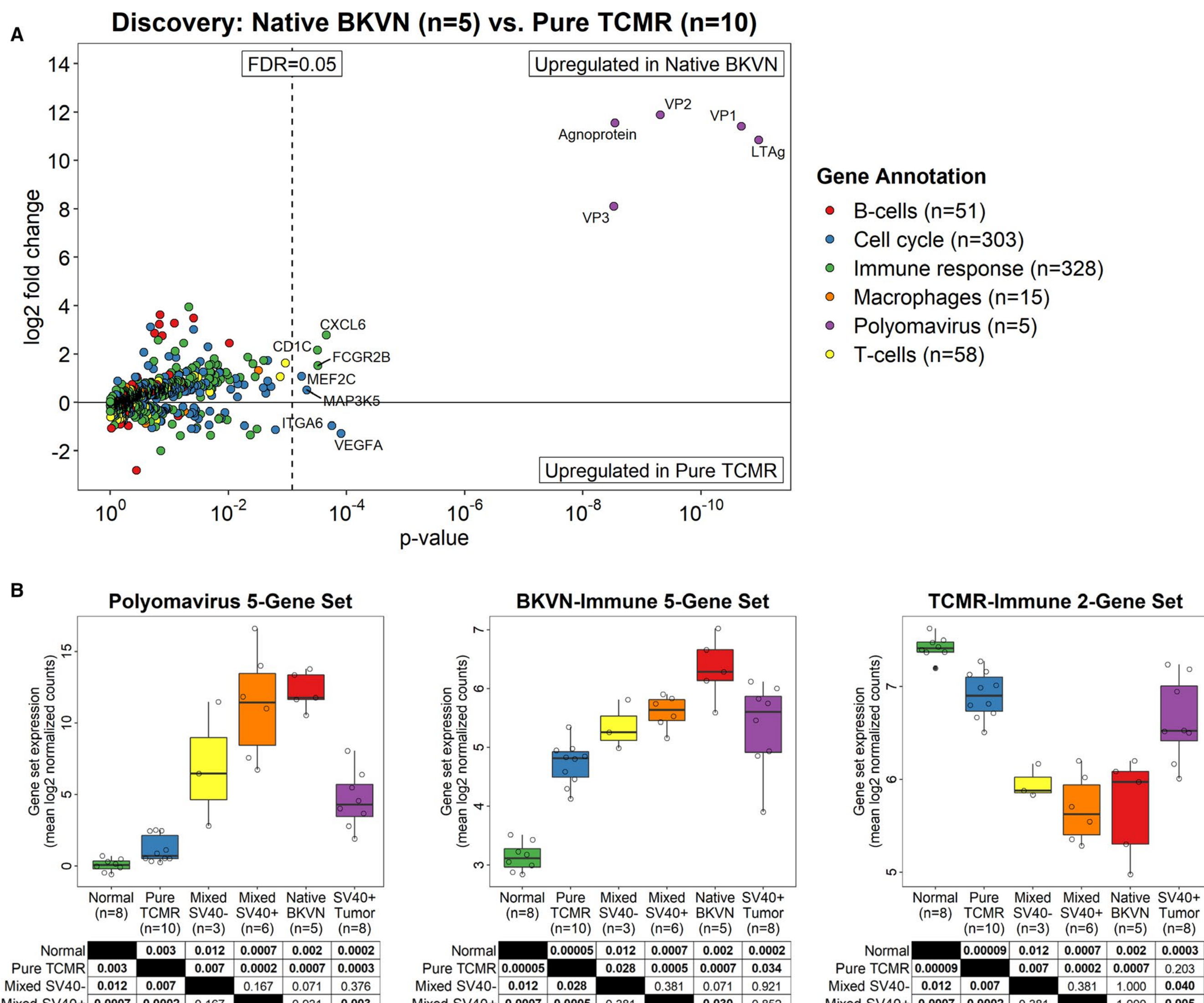

FIGURE 3 Differential gene expression between BKVN and TCMR. A, Exploratory volcano plot analysis in discovery cohort (n = 15): Out of 760 experimental genes analyzed (color coded by functional annotation), five polyomavirus genes and seven immune genes (five relatively upregulated in BKVN and two relatively upregulated in TCMR) are significantly differentially expressed between native BKVN and pure TCMR. B, Gene set expression in training cohort (n = 40): Box plots demonstrate similar patterns of gene set expression in an expanded spectrum of BK-positive (Mixed SV40−, Mixed SV40+, native BKVN, SV40 + Tumor) and BK-negative (Normal, pure TCMR) samples. TCMR-Immune 2-gene set expression is highest in Normal, indicating that relatively higher expression in pure TCMR vs the BKVN groups reflects attenuated downregulation rather than true upregulation. Boxes represent interquartile range and whiskers represent data points within 1.5× interquartile range from upper and lower box limits. BKVN, BK virus nephropathy; FDR, false discovery rate; Mixed, mixed BKVN-TCMR; SV40, simian virus 40 immunohistochemistry; TCMR, T cell–mediated rejection [Color figure can be viewed at wileyonlinelibrary.com]

nephropathy (PVN) class (rho = 0.345, P = .007), and no significant correlation with any other histology lesions (Table 3). BKVN-Immune 5-gene set expression correlated with Banff PVN class (rho = 0.535, P < .0001) as well as multiple histologic markers of acute and chronic injury, including total interstitial inflammation (rho = 0.650, P < .0001), tubulitis (rho = 0.312, P = .019), peritubular capillaritis (rho = 0.402, P = .003), interstitial fibrosis (rho = 0.634, P < .0001), and tubular atrophy (rho = 0.549, P < .0001). TCMR-Immune 2-gene set expression demonstrated only negative correlations with Banff histology lesions (consistent with it being relatively upregulated in normal and downregulated in nonnormal/injured biopsies), including the following: interstitial inflammation (rho = −0.430, P = .0009), total interstitial inflammation (rho = −0.442, P = .004), tubulitis (rho = −0.484, P = .0002), interstitial fibrosis (rho = −0.264, P = .041), tubular atrophy (rho = −0.255, P = .040), and mesangial matrix expansion (rho = −0.343, P = .012).

3.6 | Molecular risk prediction in BKVN

To assess the potential utility of intragraft gene expression testing for predicting response to treatment, the validation cohort BKVN cases

TABLE 2 Diagnostic performance of differentially expressed genes and aggregate gene sets in discovery/training cohort (n = 40) and validation cohort (n = 70)

		Discovery cohort: native BKVN (n = 5) vs pure TCMR #1 (n = 10)			Training cohort: BK positive (n = 22) vs BK negative (n = 18)		Validation cohort: allograft BKVN (n = 60) vs pure TCMR #2 (n = 10)	
Gene set/gene	**Functional association**	**Fold change**	***P*-value**	**FDR**	**AUC (95% CI)**	**Accuracy**	**AUC (95% CI)**	**Accuracy**
Polyomavirus 5-gene set					0.992 (0.976-1.000)	0.975	0.992 (0.976-1.000)	0.971
LTAg	Polyomavirus	1846.8	1.05×10^{-11}	7.86×10^{-9}	1.000 (1.000-1.000)	1.000	0.967 (0.927-1.000)	0.943
VP1	Polyomavirus	2743.6	2.07×10^{-11}	7.86×10^{-9}	0.957 (0.896-1.000)	0.925	0.985 (0.963-1.000)	0.957
VP2	Polyomavirus	3804.7	4.89×10^{-10}	1.24×10^{-7}	0.972 (0.932-1.000)	0.925	0.993 (0.979-1.000)	0.943
Agnoprotein	Polyomavirus	2991.0	2.87×10^{-9}	4.57×10^{-7}	0.932 (0.858-1.000)	0.975	0.972 (0.936-1.000)	0.929
VP3	Polyomavirus	275.5	3.01×10^{-9}	4.57×10^{-7}	0.992 (0.976-1.000)	0.925	0.933 (0.876-0.991)	0.900
BKVN-Immune 5-gene set					0.952 (0.891-1.000)	0.900	0.720 (0.499-0.941)	0.829
CXCL6	Neutrophil chemotaxis	6.9	.0002	0.021	0.919 (0.832-1.000)	0.875	0.760 (0.563-0.957)	0.757
FCGR2B	Immune complex phagocytosis	2.9	.0003	0.024	0.942 (0.873-1.000)	0.900	0.540 (0.308-0.772)	0.814
CD1C	Lipid antigen presentation to T cell s	4.5	.0003	0.024	0.833 (0.710-0.956)	0.750	0.657 (0.427-0.887)	0.771
MAP3K5	Intracellular signal transduction	1.4	.0005	0.033	0.679 (0.502-0.856)	0.725	0.550 (0.335-0.765)	0.800
MEF2C	Intracellular signal transduction	2.1	.0006	0.037	0.715 (0.553-0.877)	0.700	0.508 (0.289-0.728)	0.600
TCMR-Immune 2-gene set					0.934 (0.860-1.000)	0.900	0.633 (0.452-0.815)	0.429
VEGFA	Angiogenesis	0.4	.0001	0.016	0.816 (0.677-0.954)	0.800	0.637 (0.465-0.809)	0.471
ITGA6	Cell surface adhesion/ signalling	0.5	.0002	0.019	0.967 (0.923-1.000)	0.925	0.575 (0.386-0.764)	0.371

Abbreviations: AUC, area under the receiver operating characteristic curve; BKVN, BK virus nephropathy; FDR, false discovery rate; TCMR, T cell–mediated rejection.

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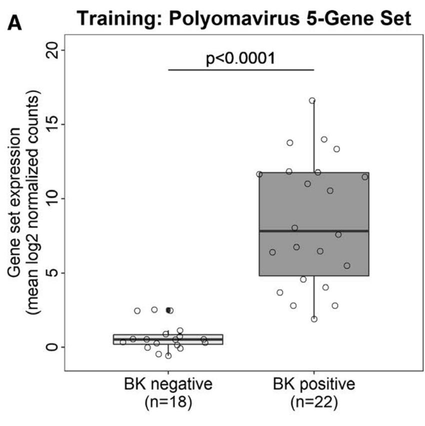

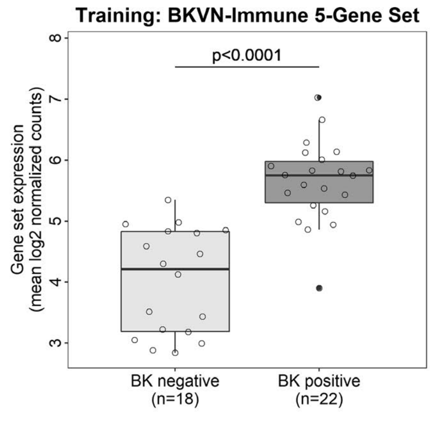

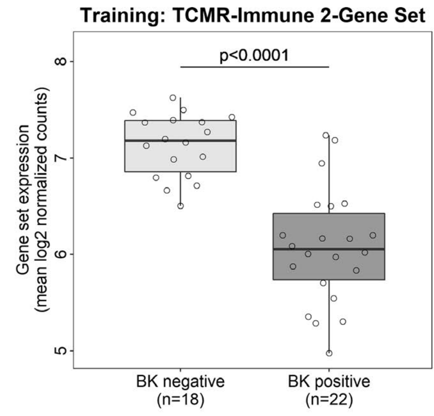

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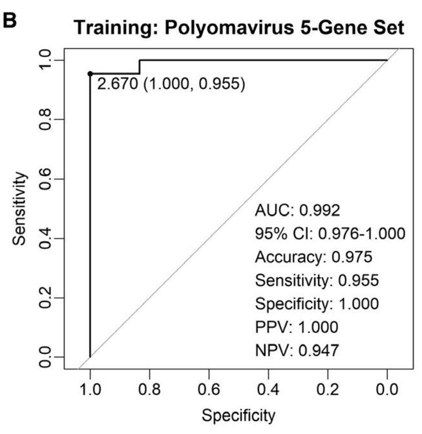

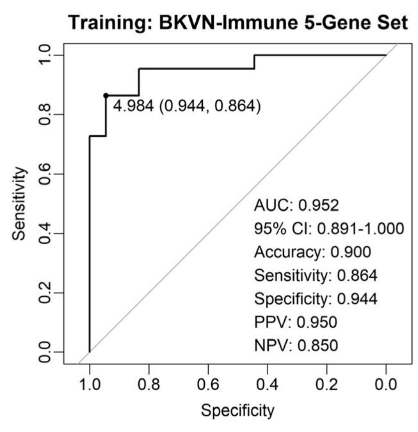

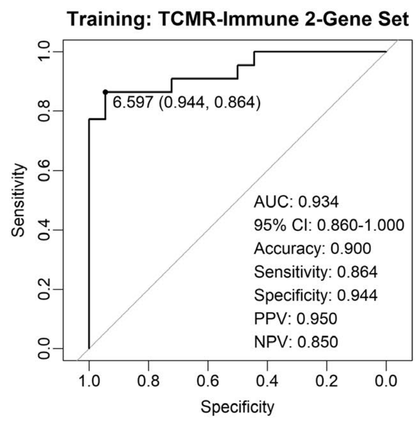

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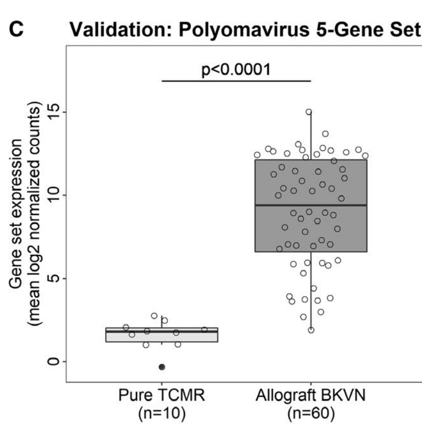

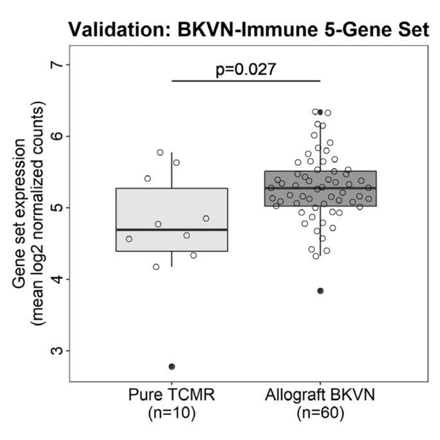

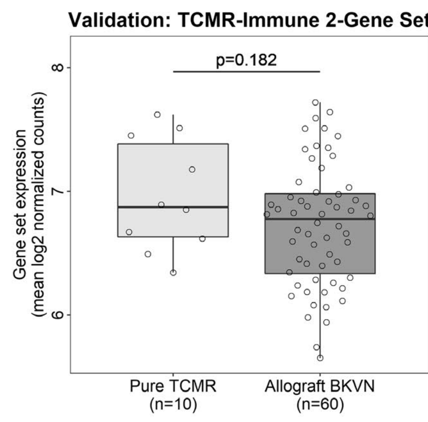

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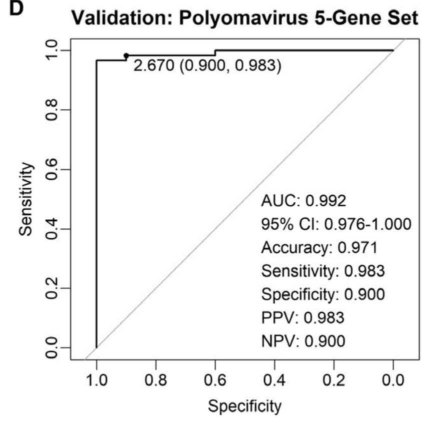

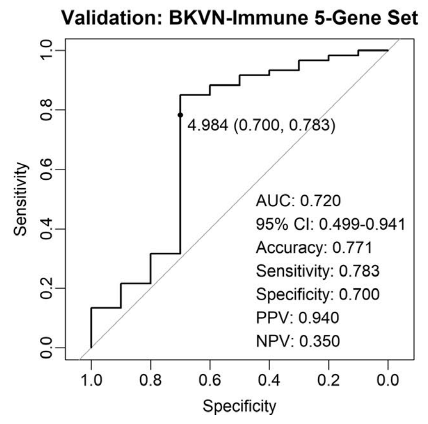

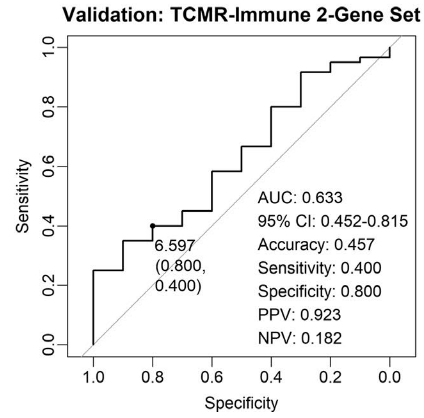

FIGURE 4 Gene set diagnostic performance in training and validation cohorts. A-B, Training cohort (n = 40): Box plots (A) and ROC curves (B) demonstrate excellent discrimination between BK-positive and BK-negative groups for all three gene sets. C-D, Validation cohort (n = 70): Box plots (C) and ROC curves (D) reveal similarly excellent diagnostic performance for the Polyomavirus 5-gene set, but suboptimal performance for the immune gene sets. Diagnostic thresholds assessed in the validation cohort were derived from the training cohort. BKVN, BK virus nephropathy; ROC, receiver operating characteristic; TCMR, T cell–mediated rejection

were categorized into one of three 6-month postintervention clinical outcome groups: Resolution (n = 35), Persistence (n = 14), or Rejection (n = 11). As expected, the Persistence and Rejection groups were associated with inferior long-term allograft survival vs the Resolution group (log-rank test, P = .005) (Figure 5A). However, separating the patients into low and high gene set expression groups revealed no significant differences in allograft survival (P = .188-0.628) (Figure 5B,D).

Exploratory volcano plot analysis was performed in an attempt to identify potential novel molecular signatures associated with each of the three clinical outcome groups, but no statistically significant differential expression was identified (FDR > 0.05) (Figure 6A). The previously identified diagnostic gene sets were therefore evaluated for potential additional utility as predictive markers. The Polyomavirus 5-gene set revealed similar expression between outcome groups (P = .240-0.979) (Figure 5B). However, TCMR-Immune 2-gene set expression was significantly higher in the Persistence group vs both Rejection (P = .001, AUC = 0.870) and Resolution (P = .0005, AUC = 0.810) groups. BKVN-Immune 5-gene set expression was modestly higher in Resolution vs Persistence (P = .042, AUC = 0.688).

To explore the potential clinical relevance of these differences in gene set expression between outcome groups, other currently available clinical and histological features were also evaluated (Table 4). The Rejection group was associated with a significantly shorter post-transplant duration than Resolution (P = .005) and Persistence (P = .006), but there were no other significant differences in clinical features at the time of biopsy, including serum creatinine, proteinuria and BK viral load, between outcome groups. Regarding histology, Persistence demonstrated slightly less interstitial inflammation than Rejection (P = .033) and less tubulitis than Resolution (P = .033), but no other significant differences in acute injury lesions were identified.

4 | DISCUSSION

Due to the inability of histopathology to distinguish renal allograft inflammation directed against viral vs allogeneic antigens, new tools are needed to help interpret the biological and clinical significance of inflammation occurring in the setting of BKVN.

TABLE 3 Correlation between gene set expression and Banff histology lesions in allograft BKVN validation cohort (n = 60)

	Polyomavirus 5-gene set		BKVN-immune 5-gene set		TCMR-immune 2-gene set	
Biopsy feature	**Spearman's rank correlation coefficient**	***P*-value**	**Spearman's rank correlation coefficient**	***P*-value**	**Spearman's rank correlation coefficient**	***P*-value**
Polyomavirus 5-gene set	N/A	N/A	0.127	.334	−0.253	0.051
BKVN-immune 5-gene set	0.127	.334	N/A	N/A	−0.447	**.0004**
TCMR-immune 2-gene set	−0.253	.051	−0.447	**.0004**	N/A	N/A
Banff PVN Class	0.345	**.007**	0.535	**<.0001**	−0.241	.064
Tissue viral load (pvl)	0.443	**.0004**	0.239	.066	−0.098	.458
Interstitial inflammation (i)	−0.129	.343	0.250	.064	−0.430	**.0009**
Total interstitial inflammation (ti)	−0.065	.690	0.650	**<.0001**	−0.442	**.004**
Tubulitis (t)	−0.015	.910	0.312	**.019**	−0.484	**.0002**
Glomerulitis (g)	−0.042	.759	0.109	.422	−0.098	.470
Arteritis (v)	−0.231	.089	0.129	.349	0.180	.188
Peritubular capillaritis (ptc)	0.151	.290	0.402	**.003**	−0.255	.071
Interstitial fibrosis (ci)	0.101	.442	0.634	**<.0001**	−0.264	**.041**
Tubular atrophy (ct)	0.153	.243	0.549	**<.0001**	−0.255	**.040**
Transplant glomerulopathy (cg)	0.086	.534	0.146	.288	−0.214	.116
Mesangial matrix expansion (mm)	−0.073	.604	0.081	.564	−0.343	**.012**
Arterial fibrointimal thickening (cv)	−0.005	.970	0.087	.543	−0.018	.901
Arteriolar hyalinosis (ah)	0.225	.092	0.062	.647	−0.021	.876

Abbreviations: BKVN, BK virus nephropathy; PVN, polyomavirus nephropathy; TCMR, T cell–mediated rejection; N/A, not applicable.

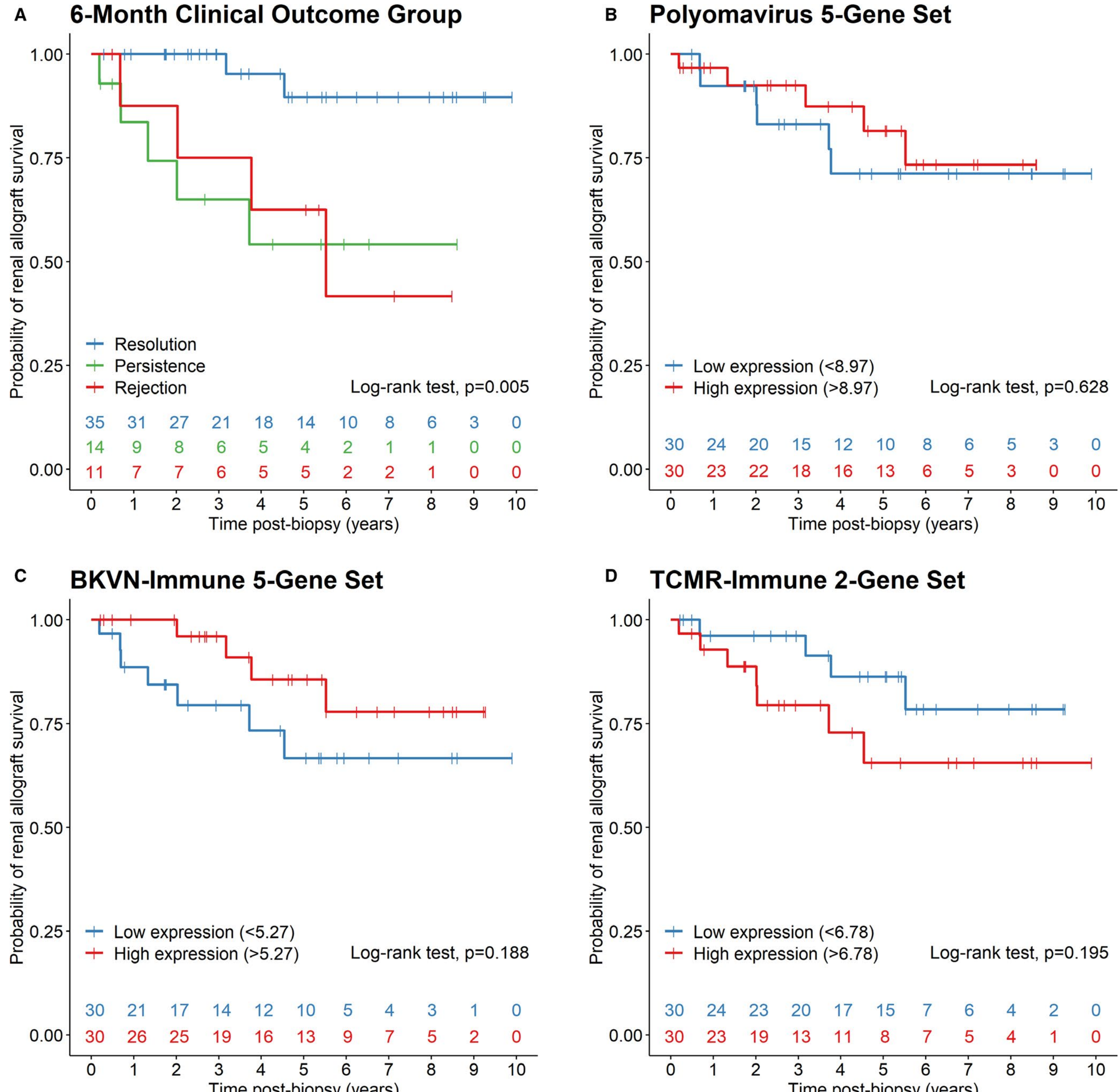

FIGURE 5 Renal allograft survival after standardized immunosuppression reduction for BKVN. A, Risk stratification by clinical outcome group: Allograft survival is significantly worse for patients with persistent viremia/BKVN or de novo rejection at 6 mo postintervention, compared with those that resolve. B-D, Risk stratification by gene set expression: Separating patients into low and high gene set expression groups reveals no significant differences in allograft survival. BKVN, BK virus nephropathy; TCMR, T cell–mediated rejection [Color figure can be viewed at wileyonlinelibrary.com]

In this study, we aimed to assess whether intragraft expression of polyomavirus genes and/or human immune genes could reliably distinguish BKVN from TCMR-related inflammation. In contrast to previous similar studies, which only compared BKVN and TCMR occurring in renal allografts, we exploited the unique advantages of the NanoString® nCounter® platform to assess rare cases of BKVN occurring in the native kidneys of non-renal transplant patients. This allowed us to evaluate a pure cohort of BKVN without the potential confounding presence of concomitant TCMR. For comparison, we utilized a cohort of pure TCMR biopsies with no clinical or histologic evidence of polyomavirus infection.

Significant differential expression was observed between native BKVN and pure TCMR for all five of the assessed polyomavirus genes and 7/755 human immune-related genes. The magnitude of differential expression observed with the polyomavirus genes was markedly greater than that seen with the immune genes. Both individually and as an aggregate gene set, the polyomavirus genes

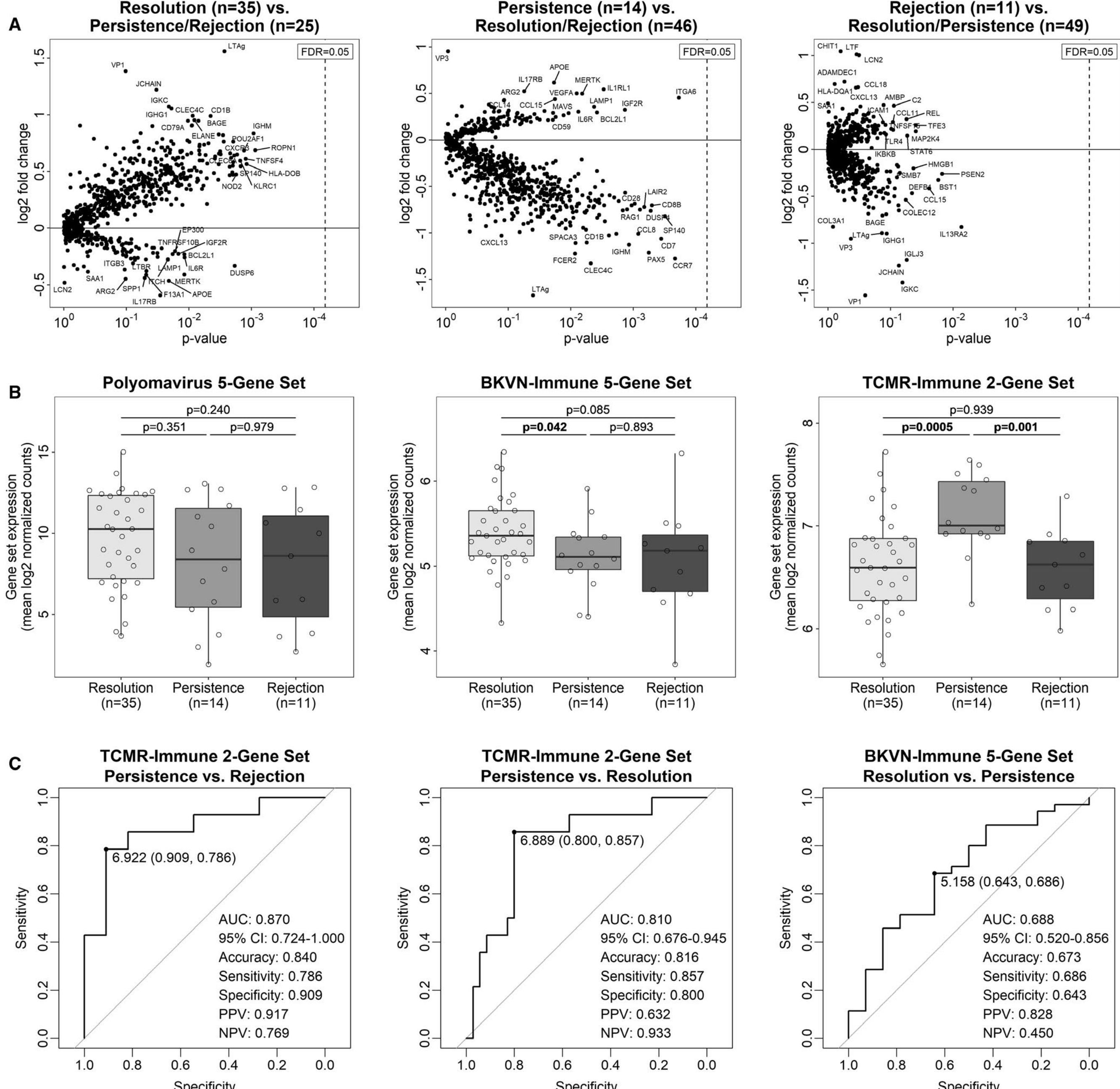

FIGURE 6 Molecular risk prediction in BKVN. A, Exploratory volcano plot analysis demonstrates no significant differential gene expression between clinical outcome groups. Top 10 up/downregulated genes are labeled for each comparison. B-C, Utility of diagnostic gene sets for molecular risk prediction: Box plots (B) and ROC curves (C) demonstrate significantly higher TCMR-Immune 2-gene set expression in Persistence vs Rejection (*P* = .001, AUC = 0.870) and Persistence vs Resolution (*P* = .0005, AUC = 0.810), as well as slightly higher BKVN-Immune 5-gene set expression in Resolution vs Persistence (*P* = .042, AUC = 0.688), but no significant differences in Polyomavirus 5-gene set expression. BKVN, BK virus nephropathy; ROC, receiver operating characteristic; TCMR, T cell–mediated rejection

demonstrated improved diagnostic sensitivity over SV40 immunohistochemistry. The Polyomavirus 5-gene set displayed similarly excellent diagnostic performance in both the training and validation cohorts (AUC = 0.992). Furthermore, it exhibited only moderate correlation with tissue viral load and Banff PVN class, and no significant correlation with any other histological lesions, which may partly be explained by gene expression analysis being more sensitive than histology. Similar levels of gene set expression were observed between SV40+ and SV40− areas of the allograft nephrectomies with mixed BKVN-TCMR, suggesting that similar molecular processes were present throughout the graft, despite regional differences in immunohistochemical staining. Although it would be feasible to translate this intragraft NanoString® gene expression methodology to less invasive samples, such as blood and urine, this would be of limited utility given the relative availability and affordability of existing PCR-based polyomavirus assays for these samples.

TABLE 4 Comparison of clinical, histological, and molecular characteristics according to 6-month clinical outcome group in allograft BKVN validation cohort (n = 60)

Feature[a]	Resolution (n = 35)[a]	Persistence (n = 14)[a]	Rejection (n = 11)[a]	*P*-value (Resolution vs Persistence)[b]	*P*-value (Resolution vs Rejection)[b]	*P*-value (Persistence vs Rejection)[b]
Clinical features (at time of biopsy)						
Patient age, yr	51 ± 14 (20-77)	54 ± 16 (22-80)	52 ± 17 (21-79)	0.376	0.918	0.476
Sex (male)	21 (60)	7 (50)	8 (73)	0.542	0.501	0.414
Time posttransplant, mo	20 ± 28 (2-154)	16 ± 12 (3-43)	5 ± 4 (1-12)	0.719	**0.006**	**0.005**
Serum creatinine, mg/dL	2.0 ± 0.8 (1.1-4.5)	2.6 ± 1.2 (1.4-4.8)	2.3 ± 0.7 (1.5-3.4)	0.246	0.185	0.839
Proteinuria, g/g creatinine	0.3 ± 0.3 (0.1-1.2)	0.4 ± 0.2 (0.2-0.7)	0.6 ± 0.6 (0.1-1.6)	0.105	0.464	0.943
BK viral load, log10 copies/mL	4.7 ± 1.2 (2.4-7.7)	5.0 ± 1.6 (2.9-9.2)	4.4 ± 0.6 (3.7-5.4)	0.969	0.310	0.291
Presence of DSA	4 (11)	3 (21)	2 (18)	0.392	0.619	1.000
Histological features						
Tissue viral load (pvl)	2.1 ± 0.8 (1-3)	2.3 ± 0.9 (1-3)	1.8 ± 1.0 (1-3)	0.483	0.344	0.240
Banff PVN class	2.0 ± 0.7 (1-3)	2.0 ± 0.7 (1-3)	1.7 ± 0.6 (1-3)	1.000	0.280	0.327
Interstitial inflammation (i)	0.9 ± 1.2 (0-3)	0.4 ± 0.8 (0-2)	1.3 ± 1.2 (0-3)	0.159	0.240	**0.033**
Total interstitial inflammation (ti)	1.6 ± 1.1 (0-3)	0.8 ± 0.9 (0-2)	1.4 ± 1.1 (0-3)	0.073	0.772	0.245
Tubulitis (t)	1.6 ± 1.4 (0-3)	0.7 ± 1.1 (0-3)	1.7 ± 1.3 (0-3)	**0.033**	0.926	0.058
Glomerulitis (g)	0.2 ± 0.6 (0-2)	0.2 ± 0.8 (0-3)	0.3 ± 0.5 (0-1)	0.573	0.418	0.283
Arteritis (v)	0.0 ± 0.0 (0-0)	0.1-0.3 (0-1)	0.0 ± 0.0 (0-0)	0.129	1.000	0.430
Peritubular capillaritis (ptc)	0.3 ± 0.6 (0-2)	0.5 ± 1.0 (0-3)	0.3 ± 0.5 (0-1)	1.000	0.770	0.852
Interstitial fibrosis (ci)	1.5 ± 1.0 (0-3)	1.2 ± 1.0 (0-3)	1.1 ± 1.0 (0-3)	0.306	0.238	0.774
Tubular atrophy (ct)	1.5 ± 1.0 (0-3)	1.1 ± 0.8 (0-3)	1.0 ± 1.0 (0-3)	0.170	0.173	0.768
Transplant glomerulopathy (cg)	0.1 ± 0.3 (0-2)	0.0 ± 0.0 (0-0)	0.0 ± 0.0 (0-0)	0.580	0.620	1.000
Mesangial matrix expansion (mm)	0.6 ± 1.0 (0-3)	0.0 ± 0.0 (0-0)	0.2 ± 0.4 (0-1)	**0.020**	0.292	0.128
Arterial fibrointimal thickening (cv)	1.8 ± 1.0 (0-3)	1.2 ± 1.0 (0-3)	1.4 ± 0.9 (0-2)	0.079	0.369	0.491
Arteriolar hyalinosis (ah)	1.3 ± 1.0 (0-3)	0.6 ± 0.5 (0-1)	0.7 ± 1.1 (0-3)	**0.041**	0.109	0.725
Banff C4d-score	0.3 ± 0.7 (0-2)	0.5 ± 0.8 (0-2)	0.2 ± 0.6 (0-2)	0.476	0.463	0.260
Gene set expression						
Polyomavirus 5-gene set	9.6 ± 2.9 (3.7-15.0)	8.2 ± 3.9 (1.9-13.1)	8.0 ± 3.8 (2.7-12.9)	0.351	0.240	0.979
BKVN-Immune 5-gene set	5.4 ± 0.4 (4.3-6.3)	5.1 ± 0.4 (4.4-5.9)	5.1 ± 0.6 (3.8-6.3)	**0.042**	0.085	0.893
TCMR-Immune 2-gene set	6.6 ± 0.5 (5.7-7.7)	7.1 ± 0.4 (6.2-7.6)	6.6 ± 0.4 (6.0-7.3)	**0.0005**	0.939	**0.001**

Abbreviations: BKVN, BK virus nephropathy; TCMR, T cell–mediated rejection.

[a]Data presented as count (%) for categorical data and mean ± SD (range) for continuous/ordinal data.

[b]*P*-values calculated using Mann-Whitney U-test for continuous/ordinal data and Fisher's exact test for categorical data.

In contrast to the polyomavirus genes, the immune transcripts determined to be significantly differentially expressed in the discovery cohort demonstrated disappointing validation cohort performance. Among the BKVN-specific immune genes reported in previous similar studies,[13,14] only *CXCL6*, a pro-inflammatory cytokine involved in neutrophil chemotaxis, displayed significant differential expression in our discovery cohort. This lack of concordance with prior literature may reflect differences in sample cohort and/or assay. It may also be due to incomplete representation of previously identified BKVN-specific transcripts in the 800-gene panel used in our study, which included only 7/14 (50%) and 66/209 (32%) of the genes reported by Mannon et al[13] and Sigdel et al,[14] respectively. However, we interpret these inconsistent and relatively low magnitude differences in immune gene expression to reflect significant molecular overlap between BKVN and TCMR-associated inflammation. This is supported by recent RNA sequencing data demonstrating significant upregulation of many previously reported BKVN-specific genes in nonviral forms of allograft injury, including TCMR, inflamed areas of fibrosis/tubular atrophy, and acute tubular injury.[15] This may be at least partly explained by observations by Zeng et al that the majority of T cell infiltrates in both BKVN and TCMR appear to represent nonspecific, secondary inflammation which is amplifying a primary antiviral and/or alloimmune response.[27] Therefore, although further analysis of such immune genes may improve our understanding of these entities, we consider them unlikely to be clinically useful for molecular diagnostic purposes with currently available platforms. However, the utility of alternative testing modalities, such as immune cell function studies, warrants further evaluation.

The second goal of this study was to explore the potential utility of intragraft gene expression for predicting clinical outcome in renal transplant patients with BKVN. Separating these patients into high and low gene set expression groups demonstrated no significant differences in allograft survival. Exploratory differential expression analysis of individual genes also revealed no significant differences between patients exhibiting resolution, persistence or de novo rejection 6 months following standardized reduction in immunosuppression. However, we did observe that the TCMR-Immune 2-gene set, initially derived for diagnostic purposes, provided significant discrimination between patients with persistent infection vs those with resolution (AUC = 0.810) or de novo rejection (AUC = 0.870). In contrast, polyomavirus gene expression was equivalent between these clinical outcome groups. Although these data suggest that it may be possible to perform molecular risk prediction in BKVN patients, this preliminary finding requires further validation in either an independent retrospective cohort or, ideally, within the context of a prospective study.

The strengths of this study include the purity of the discovery cohort, made possible by the unique ability of the NanoString® platform to analyze archival cases of native kidney BKVN. This novel approach allowed us to definitively exclude the possibility of concomitant TCMR in these biopsies. Additional strengths include the use of LCM to evaluate the sensitivity of intragraft gene expression vs histology and immunohistochemistry, although we cannot confirm whether the detected polyomavirus transcripts were present within parenchymal cells vs circulating blood within the graft. Recruitment of a relatively large, multicenter cohort of allograft BKVN biopsies with discrete clinical outcome categories also provided the unique opportunity to explore the role of molecular risk stratification in these patients.

A limitation with our strategy to analyze native BKVN biopsies is that less than 50 such cases have been reported, with even fewer having residual tissue available for retrospective molecular analysis. We attempted to mitigate this limitation by soliciting cases from a large network of global collaborators but were ultimately only able to acquire five biopsies with sufficient residual material. However, given that we were still able to identify statistically significant differences in immune gene expression, and the apparent limited utility of these genes for diagnostic purposes compared with polyomavirus genes, we believe that a larger discovery cohort would not have produced meaningfully different results. Another limitation with this study is that only 800 genes could be analyzed with the NanoString® platform, which is significantly less than the tens of thousands possible with microarrays and RNA sequencing. However, previous microarray studies have demonstrated the highly stereotyped nature of inflammatory molecular signals in allograft tissue,[11,28] and analysis of a carefully selected panel of representative genes, as in this study, is likely adequate. Furthermore, although the current analysis suggests that polyomavirus gene expression testing provides superior diagnostic sensitivity vs SV40 immunochemistry alone, we were unable to compare this with the current full clinical diagnostic approach incorporating both viremia and immunohistochemistry. In addition, although we tested SV40-negative areas from confirmed BKVN cases (which were intended to represent examples of equivocal or presumptive BKVN that were known to be truly positive), we did not directly assess gene expression in inconclusive biopsies.

In conclusion, this study provides an innovative analysis of the molecular differences and similarities between native kidney BKVN and pure TCMR, through which we demonstrated the potential utility of intragraft polyomavirus gene expression as an ancillary diagnostic for BKVN. However, due to significant molecular overlap between BKVN and TCMR-associated inflammation, our data suggest that it may not be possible to reliably evaluate for concomitant TCMR using current intragraft gene expression tools. Finally, although this study demonstrated the potential for molecular risk prediction in BKVN patients, this preliminary finding requires further validation.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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