Polyomavirus BK Nephropathy-Associated Transcriptomic Signatures: A Critical Reevaluation

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**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.

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and clinical significance of inflammation that occurs in the setting of BKPyVN. Molecular approaches have elucidated the genes upregulated in virus susceptible cells after BKV infection. 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. 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. 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. 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. 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. Five biopsies each were selected representing stable renal function (STA), ATI, TCMR, i-IFTA of undetermined etiology, and BK polyomavirus nephropathy (BKPyVN). Biopsy

| 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   |
|       | Calcinurin 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 |
|       | Calcinurin inhibitor toxicity | 49  | F   | 0     | 0      | 9      | 2.30  | 1.60  | 1.2   | Dx <12 m |
|       | Hyperuricemic nephropathy | 26  | M   | 0     | 83     | 5.79   | 3.10  | 2.30  | 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  | 1.1   | 0.97   |
|       | Hypertension     | 30  | M   | 0     | 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 |
|       | Systic lupus erythematosus | 27  | M   | 0     | 20     | 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.
designated as normal were protocol biopsies from stable patients. In the TCMR category, 1 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). 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 range 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).

**TABLE 2.** Selected genes that can discriminate TCMR from STA

| Symbol     | Entrez gene name | Location | Type(s)               |
|------------|------------------|----------|-----------------------|
| ADAMDEC1   | ADAM like decysin 1 | Extracellular space | Peptidase             |
| BTLA       | B and T lymphocyte-associated | Plasma membrane | Other                 |
| C28        | C28 molecule     | Plasma membrane | Transmembrane receptor |
| CD8A       | CD8a molecule    | Plasma membrane | Other                 |
| CXCL13     | C-X-C motif chemokine ligand 13 | Extracellular space | Cytokine             |
| IL21R      | Interleukin 21 receptor | Extracellular space | Transmembrane receptor |
| PLA2G2D    | Phospholipase A2 group IID | Extracellular space | Enzyme               |
| PTN7       | 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     | SLAM family member 8 | Extracellular space | Other                 |
| THEMIS     | Thymocyte selection-associated | Plasma membrane | Other                 |
| TIGIT      | T-cell immunoreceptor with Ig and ITIM domains | Extracellular space | 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.

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

c Shared with Table 4.

**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 Torrent 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...
determined the prealignment and postalignment quality of the reads for each sample. The aligned reads were then filtered based on read quality ($\geq 15$), alignment score ($\geq 90$), match count ($\leq 1$), mapping quality ($\geq 25$) 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 $2.0 \times$ 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.\(^6\)–\(^9\)\(^{,11\text{-}47}\) 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 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.

**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.
| 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               |
| CXL6         | C-X-C motif chemokine ligand 6        | 8.4                  | 2.2                   | <1E-45       | 7.39E-03     | Extracellular space | Cytokine            |
| CXCL13       | 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      | Nix-associated protein 1 like         | 2.9                  | 2.1                   | <1E-45       | 2.26E-04     | Plasma membrane    | Other               |
| NKAIN4C      | Na+/K+ transporting ATPase interacting 4 | 4.4               | 3.8                   | <1E-45       | 4.83E-05     | Plasma membrane    | Other               |
| PKHR         | Protein tyrosine phosphatase, receptor tyrosine phosphatase | 2.3                 | 2.8                   | <1E-45       | 3.07E-04     | Plasma membrane    | Phosphatase         |
| REGA1        | Regenerating family member 1 alpha    | 5.6                  | 2.9                   | <1E-45       | 5.84E-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             |
| ITGALC       | Integrin subunit alpha L              | 2.1                  | 2.0                   | 6.18E-14     | 5.82E-03     | Plasma membrane    | Transmembrane receptor |
| AP0C1C       | 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               |
| PTG1         | Pituitary tumor-transforming 1        | 2.5                  | 2.2                   | 7.74E-09     | 3.12E-04     | Nucleus           | Transcription regulator |
| HTRA5C       | 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         |
| LENS         | Lens epithelial protein               | 2.3                  | 2.5                   | 6.30E-06     | 2.94E-05     | Cytoplasm         | Other               |
| CSGP5        | Chondroitin sulfate proteoglycan 5    | 4.3                  | 2.4                   | 7.1E-04      | 5.02E-06     | Extracellular space | Growth factor       |
| HRP           | Haploidykin-related protein           | 2.3                  | 2.1                   | 8.30E-03     | 5.25E-05     | Extracellular space | Peptidase          |
| SAA1         | Serum amyloid A1                      | 2.5                  | 3.1                   | 3.06E-03     | 4.06E-06     | Extracellular space | Transporter         |
| HCP5         | HLA complex P5 (nonprotein-coding)    | 2.7                  | 2.5                   | 4.30E-02     | 2.52E-05     | Plasma membrane    | Other               |
| LST1         | Leukocyte-specific transcript 1       | 2.5                  | 2.4                   | 1.20E-02     | 9.78E-04     | Plasma membrane    | Other               |

*21 genes (ANKRD19P, C3, CCL18, CD48, CXL6, CXCL13, IL7R, ISG20, NCKAP1L, NKAIN4, NKG7, REGA1, SLC1A3, BIRC3, AP0C1, HTRA5, C1orf162, LENS, SAA1, HCP5, LST1) are shared with Table 4.

*Shared with Table 2 reflecting pathogenetic mechanisms in common with T cell mediated rejection.

*Shared with Table 5 reflecting significant tubular injury in both viral nephropathy and acute tubular necrosis.
### TABLE 4

**BKPvYN-specific genes upregulated in IFTA**

| 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 |
| C2L18 | C2-C motif chemokine ligand 18 | 5.4 | 2.0 | <1E-45 | 5.04E-03 | Extracellular space | Cytokine |
| C2L19 | C2-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 |
| C22orf50 | Complement component 3 | 2.6 | 2.2 | <1E-45 | 6.20E-04 | Plasma membrane | Transmembrane receptor |
| CCH6 | Cadherin 6 | 2.8 | 2.0 | <1E-45 | 6.75E-04 | Plasma membrane | Other |
| C1QC | Complement C1q C chain | 2.4 | 2.7 | <1E-45 | 2.75E-04 | Plasma membrane | Transmembrane receptor |
| ISG20 | Interferon-stimulated exonuclease 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 metalloproteinase 7 | 4.4 | 2.8 | <1E-45 | 1.24E-03 | Extracellular space | Peptidase |
| NKAIN1C | Na+/K+ transporting ATPase interacting 4 | 5.9 | 3.8 | <1E-45 | 4.83E-05 | Other | Other |
| NNM1 | 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 |
| SLAMF8b | 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 |
| LTBI | Lymphotoxin beta | 2.8 | 2.7 | <1E-45 | 4.01E-05 | Extracellular space | Cytokine |
| GPR183c | G protein-coupled receptor 183 | 3.1 | 2.1 | <1E-45 | 1.18E-04 | Plasma membrane | G protein-coupled receptor |
| SLCT3 | 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 |
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 poly-clonal 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. 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 (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.

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. 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
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. 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. 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 38500 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

### TABLE 5.

**BKPyVN-specific genes upregulated in ATI**

| Symbol      | Entrez gene name                  | Observed fold change | Published fold change | Published, P   | Location                      | Type(s)         |
|-------------|-----------------------------------|----------------------|-----------------------|----------------|-------------------------------|-----------------|
| APOC1       | 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           |
| NCAH2       | Non-SMC condensin II complex subunit H2 | 3.2                | 2.1                   | <1E-45         | 4.25E-04 Nucleus              | Other           |
| NKAIN4      | Na+/K+ transporting ATPase        | 6.7                  | 3.8                   | <1E-45         | 4.83E-05 Other                | Other           |
| GPR118      | 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       | Integrin subunit alpha L          | 2.1                  | 2.0                   | 2.53E-08       | 5.82E-03 Plasma membrane     | Transmembrane receptor |
| HOPS       | HLA complex P5 (nonprotein-coding) | 6.9                | 2.5                   | 2.07E-05       | 5.25E-05 Other                | Other           |
| HTR6        | 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          |
| C3orf16     | 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.

![FIGURE 4.](http://example.com) 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, 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.

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