Genomic Biomarkers Correlate with HLA-Identical Renal Transplant Tolerance

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ABSTRACT

The ability to achieve immunologic tolerance after transplantation is a therapeutic goal. Here, we report interim results from an ongoing trial of tolerance in HLA-identical sibling renal transplantation. The immunosuppressive regimen included alemtuzumab induction, donor hematopoietic stem cells, tacrolimus/mycophenolate immunosuppression converted to sirolimus, and complete drug withdrawal by 24 months post-transplantation. Recipients were considered tolerant if they had normal biopsies and renal function after an additional 12 months without immunosuppression. Of the 20 recipients enrolled, 10 had at least 36 months of follow-up after transplantation. Five of these 10 recipients had immunosuppression successfully withdrawn for 16–36 months (tolerant), 2 had disease recurrence, and 3 had subclinical rejection in protocol biopsies (nontolerant). Microchimerism disappeared after 1 year, and CD4+CD25+FOXP3+ regulatory T cells and CD19+IgD/M+CD27 B cells were increased through 5 years post-transplantation in both tolerant and nontolerant recipients. Immune/inflammatory gene expression pathways in the peripheral blood and urine, however, were differentially downregulated between tolerant and nontolerant recipients. In summary, interim results from this trial of tolerance in HLA-identical renal transplantation suggest that predictive genomic biomarkers, but not immunoregulatory phenotyping, may be able to discriminate tolerant from nontolerant patients.


We describe an HLA-identical tolerance trial without any myeloablation,1–4 but with four immunoselected CD34+ donor hematopoietic stem cell (DHSC) infusions in the first 9 months after renal transplantation.5–7 Temporary immunosuppression consisted of alemtuzumab induction, tacrolimus/mycophenolate converted to sirolimus, followed by complete withdrawal after a normal 24-month biopsy (Figure 1). We herein evaluate the first 10 of 20 patients enrolled, 5 of which passed the 36-month post-transplant milestone, with normal biopsies one year after complete immunosuppression withdrawal, designated as tolerant (Figure 1B and Tables 1 and 2). Two of the remaining five patients, designated as nontolerant (see clinical summaries in the Supplemental Material), had renal disease recurrence, and continued immunosuppression. The remaining three patients had (subclinical) biopsy rejection after complete withdrawal (Figure 1, C and D, and Tables 1 and 2), and immunosuppression was reinstated, solely based on the biopsies, without increase in panel reactive antibodies (PRAs) or positive donor-specific crossmatches, and with unchanged serum creatinine concentrations. The other 10 enrollees have not yet reached 36 months.

The total number of donor CD34+ purified cells infused ranged between 3.74 and 14.40×10⁶/kg recipient body weight. Only temporary PBMC chimerism was observed during the first year (not above 3%), unrelated to tolerance or nontolerance (Table 1). Prolonged CD4+ T cell depression (P<1.0×10⁻7;
Figure 2A) and parallel CD8+ changes (not shown) persisted through 60 months post-transplantation. Increased percentages of CD4+CD25hiCD127loFOXP3+ cells (phenotypic regulatory T cells; Figure 2B), some over 10 times pretransplant values ($P=0.01$), also persisted through 60 months. CD19+B cells reached more varied nadirs, but rebounded to prolonged higher levels than before transplantation ($P=0.01$; Figure 2C). Parallel increases in CD19+IgD+/IgM+CD27- (naïve) B cells also persisted through 60 months post-transplantation (Figure 2D), similar to but longer than recently described. All of the above changes occurred independent of the tolerance versus nontolerance designation. Other PBMC immunophenotypic changes (CD14, CD56, CD25, CD3/CD16) were also indistinguishable (not shown).

Figure 1. Experimental tolerance inducing protocol and representative milestone tolerant and nontolerant biopsies. (A) Planned tolerance trial protocol timeline for treatment procedures and monitoring of HLA-identical recipients up to 5 years postoperatively. (B) H&E stain of the 36-month transplant biopsy of patient 2 (Table 1) reads as normal 12 months after immunosuppression is withdrawn. This is also typical for patients 5, 6, 8, and 9 (i.e., the tolerant group) (Tables 1 and 2). (C) H&E stain of the transplant biopsy of patient 1 (Table 1) reads as Banff 1A acute rejection (with normal renal function) obtained after 12 months off immunosuppression. (D) FOXP3 stain of this biopsy. Note that the stain appears positive (arrow) in approximately 10% of the infiltrating cells (approximately 50% CD4+ cells, not shown), possibly indicative of a less severe inflammatory component. Similar findings occur in the other Banff 1A biopsies (patients 3 and 4; Table 1). However, there are very few cells to stain for FOXP3 in the tolerant recipients. H&E, hematoxylin and eosin.
Table 1. Patient demographics and clinical courses in the first 10 renal transplant recipients in the HLA-identical DHSC tolerance protocol

<table>
<thead>
<tr>
<th>Patient</th>
<th>Recipient</th>
<th>Donor</th>
<th>Preoperative Diagnosis of Native Disease</th>
<th>Peak PRA Pre-Tx (%)</th>
<th>Time Post-Tx (mo)</th>
<th>Cr (mg/dl)b</th>
<th>Latest Urine Protein per 24 h/cells</th>
<th>Latest Tx Biopsy Results (mo)</th>
<th>Current Daily IS Dose</th>
<th>Time off of IS (mo)</th>
<th>Maximum Microchimerism in Year 1 Postoperativelyc % in Blood</th>
<th>Current Microchimerism in Blood (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>51 M</td>
<td>Caucasian</td>
<td>Polycystic</td>
<td>5</td>
<td>60</td>
<td>1.2</td>
<td>0/0</td>
<td>Banff</td>
<td>Sirolimus 2 mg/d</td>
<td>12d</td>
<td>0.016</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>54 M</td>
<td>Caucasian</td>
<td>Multicystic</td>
<td>9</td>
<td>57</td>
<td>1.1</td>
<td>0/0</td>
<td>Neg: 36 (Tol)</td>
<td>0 (Tol)</td>
<td>32f</td>
<td>0.1 NT</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>46 F</td>
<td>Caucasian</td>
<td>IgAN</td>
<td>29</td>
<td>50</td>
<td>0.8</td>
<td>0/0</td>
<td>Banff</td>
<td>MMF 1 g 2×d</td>
<td>1d</td>
<td>0.1 NT</td>
<td>0</td>
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<tr>
<td>4</td>
<td>41 M</td>
<td>Hispanic</td>
<td>Type 2 diabetes mellitus</td>
<td>0</td>
<td>48</td>
<td>1.6</td>
<td>0/0</td>
<td>Banff</td>
<td>Tacro 2 mg 2×, MMF 1 g 2×d</td>
<td>12d</td>
<td>0.01</td>
<td>0.03</td>
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<td>IgAN</td>
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<td>45</td>
<td>1.4</td>
<td>0/0</td>
<td>Neg: 36 (Tol)</td>
<td>0 (Tol)</td>
<td>20f</td>
<td>2.3 NT</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
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<td>IgAN</td>
<td>0</td>
<td>45</td>
<td>1.2</td>
<td>0/0</td>
<td>Neg: 36 (Tol)</td>
<td>0 (Tol)</td>
<td>20f</td>
<td>1.7 3</td>
<td>0</td>
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<td>7</td>
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<td>Caucasian</td>
<td>Unknown</td>
<td>0</td>
<td>42</td>
<td>1.1 &lt;1 g/0</td>
<td>Disease recurrence: 18</td>
<td>Disease recurrence: 18</td>
<td>MMF 1 g 2×d</td>
<td>0d</td>
<td>0.06</td>
<td>NT</td>
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<td>11</td>
<td>41</td>
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<td>0 (Tol)</td>
<td>17f</td>
<td>0.47 8.5</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
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<td>1.4</td>
<td>0/0</td>
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<td>NT</td>
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<tr>
<td>10</td>
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<td>African American</td>
<td>Nephrosclerosis</td>
<td>15</td>
<td>36</td>
<td>2.1</td>
<td>4 g/0h,i</td>
<td>FSGS: 24i</td>
<td>MMF 1 g 2×d</td>
<td>0d</td>
<td>0.21</td>
<td>0.43</td>
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</tbody>
</table>

Tx, transplantation; Cr, creatinine; IS, immunosuppression; M, male; F, female; IgAN, IgA nephropathy; Tol, tolerant; NT, not tested; MMF, mycophenolate mofetil; Tacro, Tacrolimus.

aAt transplantation.

bMost recent serum creatinine; all values still reflect postoperative nadirs.

cPBMC and bone marrow microchimerism (up to 8.5% [patient 8] by short tandem repeat analysis) lasted up to 1 year only.
dIS reinstated, or never withdrawn (see text), but with no change in renal function; also see text (Supplemental Material) describing adverse event postponing 24-month biopsy of patient 1–30 months.

eNeg includes no acute or chronic rejection.

fTolerant indicates off immunosuppression for at least 1 year, with a normal transplant biopsy.

gNative kidney disease diagnosis was unknown (biopsies inconclusive), but possibly immune mediated, with the 18-month post-transplant biopsy showing dense deposit disease on electron microscopy.
hDisease recurrence, preoperatively undocumented.
iIntent to treat without further immunosuppression withdrawal because exclusion criteria were previously unrecognized.
differentially expressed in the nontolerant group might not make the conservative $P < 0.01$ cutoff, any differentially expressed genes with a $P < 0.05$ cutoff between healthy and nontolerant participants were subtracted from those differentially expressed between the healthy versus tolerant groups, kept at the more stringent $P < 0.001$, leaving a core set of 783 genes differentially expressed only in tolerance (Figure 3A). Notably, 780 of 783 genes were downregulated in the tolerant versus healthy participants, despite none of the tolerant participants being on immunosuppression. The majority of participants ($n=512, 66\%$) were also downregulated in the tolerant versus the nontolerant (immunosuppression) patients. A heat map of these 783 genes is shown in Figure 3B for each subject by all groups. Mapping of the top functional pathways revealed 16 significant pathways (Figure 3B). Function of these downregulated genes included 66 immune/inflammatory and cytokine genes as well as growth factors, kinases, transcription factors, and G protein–coupled receptors linked to immunity and inflammation.

The urinary cell mRNA levels in the five tolerant patients trended 2- to 3-fold lower (despite not being on any immunosuppression for at least 12 months) than the nontolerant group still on therapy (Table 2), and were not statistically significant, except for CD25 mRNA ($P = 0.01$), and were almost significant for granzyme B mRNA ($P = 0.06$).

Lifetime immunosuppression remains necessary even in HLA-identical renal transplantation, despite recent reports that late dose reductions might result in stable function, or that "spontaneous operational tolerance" may occur without deliberate attempts to induce it. There are presently three renal transplant tolerance trials that have used "nonmyeloablative" conditioning and DHSC1–4 that have resulted in both transient and permanent multi-lineage chimerism. Two trials showed that in HLA-identical pairs, complete withdrawal was successful in two thirds with stable mixed chimerism in one third. In our HLA-identical
Figure 2. T and B cell monitoring. (A) Prospective monitoring of recipient CD4\(^+\) T cell immunophenotyping for at least 36 months (up to 60 months) postoperatively. Preoperative values have all been normalized to 1. Note that with one exception (outlier) in each group in these first 10 of 20 enrollees, there is a profound and continuing depression in both the nontolerant group (solid lines) and tolerant group (dashed lines), indistinguishable from each other. The vertical axis is logarithmic. The depression actually occurred within 1 week postoperatively, but is recorded here at 3 months and then at 6-monthly intervals. (B) Prospective monitoring of recipient immunophenotypic regulatory T cells (CD4\(^+\)CD25\(^{high}\)CD127\(^{-}\)FOXP3\(^+\) cells) for at least 36 months postoperatively. Preoperative values have all been normalized to 1. Note the prolonged increase in the percentages of this subset lasting up to 60 months postoperatively, indistinguishable between nontolerant (solid lines) and tolerant (dashed lines) groups. Vertical axis is logarithmic. These percentages could only be measured from 3 months onward because of the dearth of CD4\(^+\) T cells before that time. (C) Prospective monitoring of recipient CD19\(^+\) B cells (pan-B cell epitope) immunophenotyping for at least 36 months (up to 60 months) postoperatively. Preoperative values have all been normalized to 1. Note that the early depression, although similar to the CD4\(^+\) subset, is neither as marked nor as prolonged, and that there is an overshoot above preoperative values in both the nontolerant (solid lines) and tolerant (dashed lines) groups that is indistinguishable. Vertical axis is logarithmic in each figure. Although not indicated, these changes appeared earlier as also described in A. (D) The early conversion from preoperative values, reflecting the expected mixture of naïve and activated/memory B cells, to postoperative values favoring naïve B cells. Preoperative mean ratios of 7:1 naïve/activated/memory B cells changed to postoperative mean ratios of 20:1. This
study, prolonged immunoregulation deliberately induced by DHSC infusions, alemtuzumab induction, and temporary sirolimus allowed planned immunosuppressive withdrawal without chimerism in 5 of the first 10 patients enrolled.

Ex vivo tolerance assays are challenging,1,13,14 especially with HLA identity,15 and high zone chimerism may represent the most predictive biomarker.14,16 However, HLA-identical pairs might be unique for genomic interpretation because these would not be complicated by the noise of MHC-linked immune response polymorphisms (i.e., polymorphic responsiveness against disparate donor HLA antigens being eliminated).17,18 In this respect, no (early) tolerance correlation occurred with individual A, B, C, DR, DQ, or DP HLA loci.

Although this is an interim report, our biomarker data give credence to the notion that genomic signatures may predict tolerance, and reflect tolerance once off immunosuppression. Thus, our sequential 3-year genomic PBMC analysis appears to discriminate between tolerant and nontolerant patients at 1 year (patients on sirolimus, only 1095 genes and 65% of inflammatory genes downregulated in tolerant patients), and at 2 years (when tolerant patients were weaned off immunosuppression—857 genes and 4 pathways), with 69 genes shared between the two time points. These results suggest an evolving molecular signature that may signal both successful drug weaning and a tolerance outcome.

We also identified a tolerance signature 1 year off immunosuppression comprising 783 genes, 780 of which were significantly downregulated compared with both normal healthy controls and nontolerant patients despite the latter being on immunosuppression.

These early results suggest two intriguing hypotheses. The first is that the tolerant patients, even in the absence of chimerism, are naturally immunosuppressed by a regulatory activity resembling regulatory T cells and/or regulatory B cells,19–21 not detected by the immunophenotyping thus far described. Alternatively, the tolerant patients may have a form of relative immunodeficiency...
created by the DHSC infusion protocol, not evident in the nontolerant group. In addition, with tolerance signature genes significantly downregulated compared with the nontolerant patients, this suggests that our current immunosuppressive drugs may not adequately suppress important immune/inflammatory pathways, with further implications regarding long-term allograft dysfunction and chronic rejection. Notably, the signature at 2 years, just after complete immunosuppression withdrawal, is significantly different from the signature 1 year later, with the final state of tolerance speculatively evolving in distinct stages over the 3-year time frame studied.

Consistent with PBMC microarray profiles, urinary cell mRNA levels trended lower in the tolerant (no immunosuppression) versus the nontolerant group (immunosuppression), despite the small number of participants profiled. The CD25 mRNA, a marker of activated T cells, was significantly lower, and several additional mRNAs previously associated with acute cellular rejection (CD3ε, granzyme B, perforin, IP-10, CXCR3, and CD103) all trended lower. Although levels of mRNA for tolerance markers (FOXP3, CTLA-4, TGF-β1, IL-10, and CD20) were numerically higher in the nontolerant group on immunosuppression,12 these have also been associated with rejection.22,23 Yet, none of the five patients designated nontolerant had graft...
dysfunction and might easily have been classified as “operationally tolerant,” without protocol-mandated biopsies.

Limitations of this study include the interim nature of the report with only 10 of 20 enrolled patients reaching the phenotypic milestone, and small sample sizes constituting only preliminary discovery sets for functional genomic profiling. Moreover, the two patients with recurrent disease who were considered nontolerant might be tolerant to histocompatibility antigens but still have immunopathology of their original renal disease, especially absent chimerism.24 Despite these shortcomings, we believe that this report is warranted because of the strong suggestion that predictive genomic biomarkers may be able to discriminate tolerant from nontolerant patients, absent either chimerism, or correlation with immunoregulatory phenotyping.

CONCISE METHODS

Informed Consent and Inclusion/Exclusion Criteria
Informed consent was obtained under joint supervision of Northwestern University and the Jesse Brown Veterans Affairs Medical Center Institutional Review Boards, an extramural Data Safety Monitoring Board, the National Institute of Diabetes and Digestive and Kidney Diseases sponsor, and a US Food and Drug Administration—approved investigational new drug application. Inclusion criteria included primary renal transplant recipients aged >18 years, and excluded conditions in which recipient renal disease recurrence was considered likely, and recipients with PRAs >20% by either cytotoxicity or (more commonly) flow cytometry. All had negative crossmatches against their donors.

Immunosuppression and Withdrawal
The immunosuppressive protocol (Figure 1A) consisted of two intravenous doses of 0.3 mg/kg of Campath (alemtuzumab), the first intraoperatively and the second on day 4 postoperatively. Early maintenance therapy consisted of FK506 (tacrolimus) to obtain trough levels of 8–10 ng/ml and 500 mg of concomitant mycophenolate mofetil twice daily dosed to maintain at least a minimal nucleated white blood cell count of >700/μl. At 3 months post-transplantation, tacrolimus was replaced by sirolimus (trough levels of 8–10 ng/ml). Mycophenolate was discontinued between 12 and 18 months and sirolimus was discontinued (complete immunosuppressive withdrawal) by 24 months post-transplantation.

HLA Typing and Antibody Assays
Confirming HLA identity and flow cytometric crossmatching included the use of sequence specific oligonucleotide probe hybridization molecular typing for HLA A, B, C, DR, DQ, and DP alleles. When possible, genotypes were confirmed with parents of the sibling pairs. Solid phase-based PRA testing was performed using Labscreen flow beads (One Lambda Inc., Canoga Park, CA). Donor-specific flow cytometric crossmatching and cytotoxicity PRA screening were performed cooperatively and yearly post-transplantation as previously described.25

Recipient DHSC Infusion Protocol
The DHSC protocol consisted of four infusions. The first DHSC product was obtained intraoperatively by bilateral aspiration of approximately 750 ml of donor iliac crest bone marrow after the donor kidney had been removed and before cessation of anesthesia.
From this product, CD34+ selection and immediate cryopreservation were performed. Then DHSC infusions into the recipients were given on postoperative day 5, 1 day after the second alemtuzumab dose (Figure 1). The number of cells infused varied between 0.3 and 1.0 x 10^6 cells/kg recipient lean body weight. Three months post-transplantation, the donors underwent two successive daily leukaphereses for CD34+ Neupogen mobilized DHSC immunoselection from peripheral blood. From the first day’s leukapheresis product, immunoselected CD34+ cells were infused into the recipient freshly that day. From the second day’s product, the immunoselected CD34+ cells were cryopreserved in two equal aliquots and then infused at 6 and 9 months postoperatively, respectively. At least 0.7 x 10^6 cells/kg recipient body weight were infused each time, containing at least 100-fold fewer CD3+ cell contaminants.

**Chimerism Testing**

Chimerism analysis was performed by Dr. David Senitzer of the Gift of Hope Medical Center (Duarte, CA) and in the Histocompatibility Laboratory of the Northwestern Comprehensive Transplant Center. This consisted of real-time PCR using tandem repeat sequences of DNA isolated from recipient cryopreserved PBMC and iliac crest bone marrow aspirations obtained sequentially at 3, 6, and 12 months postoperatively using (nonchimeric) donor and recipient PBMC obtained preoperatively as standards. The sensitivity of the assay was 0.001%.

**Flow Cytometry Analyses**

Recipient heparinized peripheral blood and iliac crest bone marrow aspirates were labeled with mAbs for membrane epitope detection of PBMC and bone marrow subsets and intracellular detection of FOXP3. The labeled membrane epitopes were for T cells and subsets (CD3, CD4, CD8), and CD127) or activation markers (CD25 and CD28), for B cells and subsets (CD19, IgM/IgD, CD5, and CD27), and for monocytes (CD14) and natural killer cells (CD56), all directly conjugated with one of the following five fluorochromes: FITC, phycoerythrin, electron-couple dye, phycoerythrin-cyanin 5, and phycoerythrin-cyanin 7 (all from Beckman-Coulter, Miami, FL). The FOXP3 monoclonal reagent was obtained from e-Bioscience (San Diego, CA).

Red cells were treated with lysing solution (Beckman-Coulter) followed by washings. Then 1 x 10^5 cellular events were acquired on a five-color FC 500 flow cytometer. For intracellular FOXP3 detection, Ficoll gradients were first used to isolate mononuclear cells.

**Biopsy Processing**

Protocol transplant biopsies were obtained preimplantation, at 12, 18, 24, and 36 months postoperatively, or at other times for cause; stained routinely using hematoxylin and eosin, periodic acid-Schiff, and Trichrome; and when indicated, immunostaining and electron microscopy were used.

**Peripheral Blood Genomics (mRNA Analyses)**

A total of 46 PBMC samples representing blood draws from four time points in the first nine recipients were processed for microarray analysis (The Scripps Research Institute, La Jolla, CA). The analyzed time points were as follows: immediately preoperatively in the absence of immunosuppression (n=9), postoperatively at 1 year (n=8; range, 11–13 months), at 2 years (n=12; range, 18–25 months), and >3 years (n=17; range, 32–48 months). To note, at year 2 and at >3 years, repeated samples were obtained from individual participants; at 1 year, one participant had a technically unsatisfactory sample. To discount the effects of immunosuppression on gene expression, microarray data were included on whole blood from 18 healthy human participants (controls: GSE40586; NCBI Gene Expression Omnibus [GEO] repository). All samples were prepared using standard Affymetrix protocols and analyzed on Hu Gene 1.1 ST microarrays, composed of exon-level probe sets for the entire human transcriptome. CEL files from all samples (n=46 from study participants, n=18 from controls) were normalized using the Robust Multichip Average and the normalized signals were analyzed for significant differential gene expression signatures by ANOVA, including multiple corrections testing using Partek Genomics Suite 6.6. Ingenuity Pathway Analysis software was used to map the genes to functional pathways. The gene expression results presented in this manuscript are available from the GEO repository using accession number GSE45593.

**Urinary Cell mRNA Profiling**

Urine samples (approximately 50 ml) were collected and cell pellets were prepared with the use of a standard protocol for urine cell sedimentation, stored at −80°C and shipped to the Gene Expression Monitoring (GEM) Core at Weill Cornell Medical College (New York, NY). Total RNA was isolated from the pellets using a commercial kit and the quantity (absorbance at 260 nm) and purity (ratio of the absorbance at 260 and 280 nm) of the isolated RNA were measured using the NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific). A RNA sample was classified as quality control passed if the 18S rRNA copy number was ≥5 x 10^5 and its TGF-β1 mRNA copy number was ≥100 copies in 1 μg of RNA. The total RNA was reverse transcribed to cDNA using the TaqMan RT kit (cat. N808-0234; Applied Biosystems) on the same day. The GEM Core designed gene-specific oligonucleotide primers and TaqMan fluorogenic probes (hydrolisis probes) were used for the measurement of levels of miRNAs, 18S rRNA, and BKV-VP-1 mRNA in the real-time quantitative PCR assays using a two-step process (preamplification step followed by measurement of miRNAs with an ABI Prism 7500 fast detection system; 18S rRNA and BKV-VP-1 mRNA were measured without preamplification with gene-specific primers). Absolute levels of miRNAs were calculated using the standard curve method and transcript abundance was reported as mRNA copies per 1 μg RNA. The GEM Core was blinded to any clinical information and as to tolerant or nontolerant status until PCR assay results were reported to the clinicians.

**Statistical Analyses**

Data were analyzed as the mean ± SD or SEM. Parametric (paired t tests) and nonparametric (Mann–Whitney U test/Wilcoxon signed-rank tests) were used among compared groups. Significance was established at two-sided α levels of 0.05 using statistical software (SAS Inc., Cary, NC).

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DISCLOSURES

None.

REFERENCES


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