BK virus RNA can be detected in archival renal transplant biopsies using the reverse transcription polymerase chain reaction

Kathryn J. Wiggins1,2, Renae M. Gow1, John Kanellis3, Prue Hill4, Darren J. Kelly1, Alison Skene5, David J. Goodman2 and Robyn G. Langham1,2

1Department of Medicine, St Vincent’s Hospital, University of Melbourne, Clinical Sciences Building, Cnr Princes and Regent Streets, Fitzroy Victoria 3065, 2Department of Nephrology, St. Vincent’s Hospital, PO Box 2900, Fitzroy Victoria 3065, 3Department of Nephrology, Monash Medical Centre, 246 Clayton Road, Clayton Victoria 3168, 4Department of Anatomical Pathology, St. Vincent’s Hospital, PO Box 2900, Fitzroy, Victoria 3065 and 5Department of Anatomical Pathology, Monash Medical Centre, 246 Clayton Road, Clayton Victoria 3168, Australia

Keywords: archival renal biopsy; BK virus; PCR; renal transplant; RNA

Introduction

BK virus (BKV) nephropathy (BKVN), also known as polyoma virus nephropathy (PVAN), is a significant cause of allograft dysfunction and loss in renal transplant recipients. Early reports of this condition suggested that the rate of graft failure due to BKVN was 50–80% [1–3]. Since the publication of these reports, increased awareness and progressively widespread availability of PCR assays to detect viral DNA in urine and blood have led to early recognition and diagnosis of this condition [4]. However, a contemporary case series of patients who received transplants in a centre where PCR was used as a screening tool still reported major decline in graft function in 24% of affected individuals, and graft loss in 15% [5]. Initial treatment for BKVN involves reduction of immunosuppression. Although generally well tolerated, this is associated with a risk of subsequent acute rejection (AR) [6,7]. These findings suggest that a role exists for additional diagnostic tests that could be used in conjunction with currently available methods to enhance the specificity and sensitivity of diagnosis.

Schmid et al. suggested that detection of BKV RNA in renal biopsies by real time polymerase chain reaction (RT-PCR) would provide a sensitive screening tool for intrarenal BKV replication [8]. Until recently studies of RNA expression have required macrodissection of renal cortex and storage in special facilities at the time of renal biopsy so as to provide fresh tissue for analysis [9]. While these measures give a high yield of RNA, they preclude measurement of RNA expression in the routine analysis of renal biopsies.

More recently, new methodologies have permitted RNA extraction from formalin fixed, archival tissues, including renal biopsies [10]. This has led to the study of RNA expression from archival tissue as a routine diagnostic tool in some fields [11]. RNA extracted from archival tissue yields comparable information about gene expression to RNA obtained from fresh tissue [12]. This raises the possibility of introducing evaluation of RNA, including measurement of BKV RNA, as part of the routine analysis of renal biopsies. In this study, we retrospectively performed RT-PCR in sets of serial biopsies from patients diagnosed with BKVN. Our aim was to evaluate whether BKV RNA could be consistently detected in biopsies from patients with BKVN. We also explored the potential role of RT-PCR in the diagnosis of BKVN.

Materials and methods

Subjects

Six renal transplant recipients who had been diagnosed with BKVN, and in whom serial post-transplant biopsies were available, were identified. The patients had undergone transplantation at St Vincent’s Hospital or the Monash Medical Centre between February 2000 and December 2005. In all cases, the diagnosis of BKVN was determined by biopsy findings of viral inclusions and positive SV40 antigen immunostaining, with or without accompanying inflammatory infiltrates, tubulitis and interstitial fibrosis. Serum and urine PCR for BKV DNA was performed at the time a diagnosis of BKVN was made to confirm the presence of BKV infection.

Biopsies were also obtained from three patients with BKV viraemia without histological evidence of BKV infection, and from nine renal transplant recipients with no serological or histological evidence of BKV infection; five
of these were protocol biopsies. In total, biopsies from 18 patients were studied. Eleven implantation biopsies (nine live donor, two deceased donor) were used as normal control tissue. Clinical and demographic data were obtained from patients’ medical histories.

Renal biopsies

Implantation biopsies were taken immediately following release of the vascular cross-clamps. Post-transplant biopsies were performed when considered clinically appropriate by the treating physician. In addition, since 2006, protocol biopsies from patients with stable allograft function have routinely been performed 3 months after a transplant at St Vincents’s Hospital. When performing biopsies, the transplant kidney was localized by ultrasound. A 14-gauge spring-loaded needle was used to take two to three cores of renal transplant tissue. Cores were placed in normal saline, and then transported to the pathology department. Samples were fixed in formalin within 4 h and embedded in paraffin blocks. Blocks were stored in the pathology archive department in a dust-free environment at room air.

RNA extraction

Prior to July 2007, RNA was extracted from scraped sections of paraffin blocks by the phenol–chloroform method. In brief, two 4-μm paraffin sections were cut on a microtome and placed into sterile microfuge tubes and stored In brief, two 4-

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**RNA extraction**

Prior to July 2007, RNA was extracted from scraped sections of paraffin blocks by the phenol–chloroform method. In brief, two 4-μm paraffin sections were cut on a microtome and placed into sterile microfuge tubes and stored at −80°C. The first sections of the block obtained were discarded so as to ensure that intact RNA was used in the experiment. Sections were deparaffinized by immersion in xylene followed by three washes with 100% ethanol. They were then resuspended in 200 μL of lysis buffer containing 10 mmol/L Tris/HCl (pH 8.0), 0.1 mmol/L ethylenediaminetetraacetic acid (pH 8.0), 2% sodium dodecyl sulfate (pH 7.3) and 500 μg/mL protease K (Sigma, Deisenhafen, Germany) and incubated at 60°C for 16 h.

RNA was extracted by chloroform and phenol extractions. Two hundred microlitres of chloroform and water-saturated phenol was added to each sample that was vortexed, and then centrifuged for 5 min at 4°C. The supernatant was removed and a further chloroform/phenol extraction was performed, followed by a further extraction with chloroform alone. RNA was precipitated with 180 μL of isopropanol, 19 μL of 3 mol/L sodium acetate (pH 5.2) and 1 μL of 20 mg/mL of carrier glycogen at −20°C. In the final step, samples were centrifuged for 15 min at 4°C and the supernatant was removed. The RNA pellet was washed with 1 mL cold 70% DEPC-treated ethanol and centrifuged for 10 min at 4°C. The RNA was resuspended in 10 μL of DEPC-water and stored at −80°C [10].

From July 2007, RNA was extracted using a commercially available kit, RNEasy FFPE (Qiagen GmbH, Hilden, Germany) [13]. Samples were processed according to the manufacturer’s directions. This method yields RNA equivalent in quality and quantity to that obtained using the phenol–chloroform method (unpublished data, Kate Wiggins).

Eight microlitres of RNA was reverse transcribed with 1 μL of random hexamers (2 μg/μL) and 3 μL of DEPC-treated water and incubated for 5 min at 70°C. After cooling on ice for 5 min, 5 μL of 5× AMV reaction buffer, 2.5 μL of 10 mM dNTP mix, 0.5 μL RNase inhibitor (40 U/μL) (Roche Diagnostics, Mannheim, Germany), 0.5 μL AMV reverse transcriptase (25 U/μL) (Roche Diagnostics, Mannheim, Germany) and 4.5 μL of DEPC water was added. The reaction mixture was incubated at 37°C for 60 min to complete cDNA synthesis after which the cDNA samples were stored at −20°C until RT-PCR was performed. DNase was not used as the yield of RNA from formalin-fixed tissue is low, and residual RNAases may lead to degradation of RNA [14].

**Polymerase chain reaction (PCR)**

PCR was performed using the ABI Prism 7000 Sequence Detector (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Reactions were performed in duplicate, and no template controls and positive controls consisting of human nephrectomy specimen were used. Ribosomal 18S was used as a housekeeping gene. The target gene for BKV was the large T sequence. Primers were obtained from Sigma-Aldrich and fluorescent probes were obtained from Applied Biosystems (Foster City, CA, USA). Primer and probe sequences are summarized in Table 1. Primers were designed to span an exon–exon boundary and thus minimized the risk of amplification of contaminating DNA. The 25-μL PCR mixture contained 12.5 μL of Taqman Universal PCR Master Mix, 500 nM primers (forward and reverse), 100 nM of Taqman probe and 2 μL of cDNA template. PCR was performed at 50°C for 2 min, 95°C for 10 min, and then run for 50 cycles at 95°C for 15 s and 60°C for 1 min. Relative gene expression was measured by calculating delta concentration threshold (ΔCt) values between test and control genes [15].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BKV large T gene sequence</td>
<td>Forward primer GAAAGCTTTAGGTCTTT&lt;br&gt;Reverse primer CTACCTT&lt;br&gt;Probe GGTGCAACCCTATGGAAC&lt;br&gt;AGA&lt;br&gt;Probe FAM-AATCTGCTGTTGCCTC&lt;br&gt;TTCATCACTGGCA-MGB&lt;br&gt;TCAGGGCTGTGTAATGGAA</td>
</tr>
<tr>
<td>18S</td>
<td>Forward primer CCCTCCAAATGGATCTCTGTT&lt;br&gt;Reverse primer VIG-AGTCCACTTTAAATCTT&lt;br&gt;Probe T-MGB</td>
</tr>
</tbody>
</table>

**Histological analysis**

Renal biopsies were assessed for the presence of BKVN by a nephropathologist. Where BKV RNA was detected retrospectively, biopsies were restudied histologically and underwent repeat immunostaining for the SV40 antigen, to look for previously undetected histological evidence of BKVN. Biopsies with BKVN were graded according to the method described by Drachenberg et al. [16].

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Table 1. Sequences of primers and probes used in the reverse transcription polymerase chain reaction

<table>
<thead>
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<tr>
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</table>
BK virus RNA in archival renal transplant biopsies

Table 2. Characteristics of 18 patients whose renal transplant biopsies were analysed. Results are expressed as median (interquartile range) for non-parametric continuous data and number (percentage) for categorical data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>All patients (n = 18)</th>
<th>Patients with BKVN (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>54 (41–58)</td>
<td>49 (41–50)</td>
</tr>
<tr>
<td>Male</td>
<td>11 (61)</td>
<td>4 (67)</td>
</tr>
<tr>
<td>Cause of end-stage renal disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>5 (28)</td>
<td>2 (33)</td>
</tr>
<tr>
<td>Reflux nephropathy</td>
<td>2 (11)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>10 (56)</td>
<td>4 (67)</td>
</tr>
<tr>
<td>ADPKD</td>
<td>1 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>6 (33)</td>
<td>2 (33)</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>2 (11)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Former</td>
<td>2 (11)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>Never</td>
<td>14 (76)</td>
<td>5 (83)</td>
</tr>
<tr>
<td>Transplant type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live donor kidney</td>
<td>5 (28)</td>
<td>2 (33)</td>
</tr>
<tr>
<td>Deceased donor kidney</td>
<td>12 (67)</td>
<td>3 (50)</td>
</tr>
<tr>
<td>Simultaneous kidney–pancreas</td>
<td>1 (6)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>Transplant number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>15 (83)</td>
<td>5 (83)</td>
</tr>
<tr>
<td>2</td>
<td>2 (11)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>3</td>
<td>1 (6)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>Basiliximab induction therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>7 (39)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>10 (55)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>Biopsy-proven acute rejection</td>
<td>6 (33)</td>
<td>4 (67)</td>
</tr>
</tbody>
</table>

*One patient was taking sirolimus instead of a calcineurin inhibitor.

Quantification of tissue fibrosis

The amount of interstitial collagen deposition was quantified using Analytical Imaging System (AIS; Ontario, Canada). In brief, non-overlapping sections of renal cortex stained with aldehyde fuschin Gomori were photographed at ×20 magnification with a microscope (AxioVision rel 4.5 Zeiss) attached to a camera (AxioCam MRC5). AIS software was used to define areas of collagen deposition (green), and the proportional area of staining was quantified.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM) for continuous parametric variables, median (interquartile range) for non-parametric continuous variables and number (percentage) for categorical variables unless stated otherwise. Differences between groups were evaluated using the Wilcoxon signed-rank test. Statistical analysis was performed using the statistical software package, SPSS for Windows release 14.0.0 (SPSS Inc., North Sydney, Australia). P values <0.05 were considered statistically significant.

Results

Patient characteristics

The patient characteristics are summarized in Table 2. There was a higher incidence of biopsy-proven AR in the patients who developed BKVN. All patients in this group received tacrolimus as part of their maintenance immunosuppression regimen. None of the patients in the BKVN group had serum or urine PCR performed prior to being diagnosed with BKVN.

BKVN in renal biopsies

Nine biopsies from six patients had histological evidence of BKVN. One biopsy showed stage A disease, two stage B1, one stage B2 and five stage B3.

BKV RNA detection

BKV RNA was detected in all nine biopsies with histological evidence of BKVN. A further six biopsies were also positive for BKV RNA. Two of these were reported as having normal morphology, and four as having AR. Retrospective immunostaining for SV40 antigen was negative in all of these biopsies. Each was performed prior to the histological diagnosis of BKVN being made. In total five of the six patients diagnosed with BKVN had at least one biopsy where BKV RNA was identified in renal tissue before a histological diagnosis of BKVN was made. The time course of BKV RNA detection and diagnosis of BKVN for the six patients with BKVN are shown in Figure 1.

BKV RNA was not detected in any renal transplant biopsies taken from patients with positive serum PCR but no histological changes of BKVN. Similarly, BKV RNA was not found in any implantation biopsies, or in biopsies from transplant recipients with no evidence of BKV infection.

Relationship between relative viral load and clinical course

The relative viral load (VL), measured by calculating relative gene expression, was calculated in one patient who had serial biopsies positive for BKV RNA. For these
calculations, the gene expression in the earliest biopsy with BKV RNA was assigned a relative value of 1.0. The VL increased from the time BKV RNA was first detected to the time of the biopsy showing histological changes of BKVN. Following reduction in immunosuppression, and stabilization of the serum creatinine, the VL fell. The VL and clinical course of the patient are shown in Figure 2.

Comparison of RT-PCR and histology

The mean time to the first biopsy with detectable BKV RNA was 100.0 ± 38.9 days compared to 300.7 ± 99.2 days to histological evidence of BKVN (P = 0.007). The serum creatinine was slightly lower at the time of the first biopsy showing RNA than at the time a histological diagnosis was made (173 ± 10.6 versus 191 ± 27.3 µmol/L). However, this difference was not statistically significant.

The amount of collagen deposition was quantified in four of six patients diagnosed with BKVN. The proportional cross-sectional area of collagen deposition was greater in the biopsies showing histological evidence of BKVN than in biopsies in which only RNA was detected [2.1 (1.0–4.2) versus 12.9 (11.79–23.82, P = 0.04). Representative photomicrographs of biopsies from a patient in the cohort are shown in Figure 3. These clearly demonstrate increased areas of fibrotic change in the biopsy with diagnostic changes of BKVN compared to the earlier biopsies when RNA was first detected. Colour images of the aldehyde fuschin Gomori stained sections, in which areas of fibrosis appear green, are available as online supplementary material.

Discussion

In this study, we have demonstrated that RT-PCR can be performed on routinely processed renal transplant biopsies taken from patients with BKVN to detect BKV RNA. While this technique clearly requires further investigation, particularly in conjunction with serum PCR testing, the findings raise the possibility that it may have a role as a diagnostic test in conjunction with currently used methods.

Increased recognition, better understanding of management and widespread use of serum PCR assays have led to a reduction in graft loss due to BKVN from 50 to 80%, to 15% [2,5,17]. However, BKVN continues to be a significant cause of renal transplant dysfunction and graft failure. Initial treatment of BKVN consists of reduction in immunosuppression, which is generally well tolerated but carries a small risk of invoking later AR [6,7]. Performing PCR on serum is a sensitive method of detecting patients at risk of BKVN. However, less than half of the patients with positive serum PCR develop BKVN [6]. Use of RT-PCR to detect viral RNA in biopsy tissue, as in this paper, has the potential to identify the subgroup of patient with BKV viraemia in whom intrarenal viral replication is present.

In this study, we measured BKV RNA rather than DNA. As BKV is a DNA virus that remains latent in uroepithelial cells following primary infection [18], viral DNA may be present in the setting of latent infection. This has been demonstrated previously by authors who detected BKV DNA in 30–50% of native renal biopsies taken from healthy subjects. [19,20]. An important step in the pathogenesis of BKVN is viral replication, which involves transcription of DNA into RNA [21]. Therefore, viral RNA will be present
only when the BKV is actively replicating, in contrast to DNA, which is present in both latent and active infection. For this reason, we believe that measurement of RNA, rather than DNA, is a reliable method of detecting active infection at an early stage prior to the development of histological changes of nephropathy. It was beyond the scope of this study to test for BKV DNA in renal biopsies, but doing so would help confirm the proposed superiority of testing RNA rather than DNA.

We demonstrated that BKV RNA was present in all biopsies with histological evidence of BKVN, but was absent from all control tissue (implantation biopsies and post-transplant biopsies from patients with no evidence of BKV infection). These findings suggest that this methodology is both sensitive and specific. Furthermore, this technique was performed on renal transplant biopsies that were performed and processed in a standard fashion, indicating that this methodology has the potential to be applied in the routine analysis of renal transplant biopsies. Of note, the initial method used for RNA extraction in this study is time consuming. However, as mentioned in the Materials and methods section, our laboratory has started using an alternative method that takes only a few hours but yields similar amounts of RNA, increasing the feasibility of this approach.

An additional finding in this study was that in five of six patients who developed BKVN, BKV RNA was present in at least one post-transplant biopsy taken prior to the diagnosis of BKVN being made. In contrast, BKV RNA was not found in biopsies from three patients with positive serum PCR but no histological evidence of BKV infection. All of these three patients have stable graft function at least 2 years after the positive serum PCR result, with no evidence of BKVN. These findings raise the possibility that renal biopsy RT-PCR permits earlier diagnosis of BKVN than by using histology alone, while differentiating between patients who have intrarenal infection and viraemia alone. When compared to the biopsy diagnostic of BKVN, the first biopsy with BKV RNA was taken earlier, and had less interstitial fibrosis present, both of which are positive prognostic factors in BKVN [16,22,23].

Other groups have also considered detection of BKV RNA and DNA in renal biopsies as a diagnostic tool. Schmid et al. reported that BKV RNA was isolated from eight renal transplant biopsies that also showed histological changes of BKVN and from one biopsy without histological changes [8]. In contrast to the present study, subsequent biopsies from this latter patient were not available, so the authors were unable to explore whether BKVN developed at a later stage. Of note, however, they did reduce immunosuppression on detecting the BKV RNA that may have modified the disease course. Randhawa et al. measured BKV DNA in renal biopsies from non-immunosuppressed patients and transplant recipients with BK viruria [24]. As discussed previously, tests for DNA do not differentiate as reliably between active and latent infection, so are less useful.

The results of this study are preliminary and clearly require further investigation. Interdisciplinary guidelines written by Hirsch et al. [25] recommend performing urine PCR studies as a screening test at regular intervals or in the event of allograft dysfunction. Positive urine tests require confirmation within 4 weeks and correlation with other tests such as serum PCR. Incorporation of testing for BKV RNA in renal biopsies may be a useful adjunctive test, particularly in cases where differentiation between AR and BKVN is required.

A major limitation of this study was that serum and urine BKV PCR was not performed in the patients with BKVN until after BKVN was diagnosed. Clearly this precludes comparison of the two techniques in assisting early diagnosis. An additional issue is that BKV nephropathy is often focal; therefore, as with histological studies, performance of RT-PCR may be subject to a sampling error. The retrospective nature of the study predisposed to selection and ascertainment biases. Our results were consistent within the study population; however, the sample number was very small, reducing the applicability of the results to a larger population. A vintage effect may also be important. A lower threshold of suspicion for the presence of BKVN combined with the widespread availability of screening tests such as serum PCR may have already led to a general increase in early diagnosis compared to a couple of years ago when some of the patients in our cohort were diagnosed with BKVN.

Many of the limitations discussed could be addressed by prospective studies in a larger population. Such studies should include development of quantitative assays and correlation of quantitative assays of tissue RNA and DNA with serum and urine DNA assays and clinical outcomes. Measurement of recipient, and when possible, donor anti-BKV antibodies would also be of value.

We did not perform DNase treatment on RNA extracted using the phenol–chloroform method, as we believed it may reduce the yield of RNA and that the primer pairs used spanned exon–exon boundaries, thereby reducing the risk of amplification of contaminating DNA [14]. However, formal testing of DNase treatment prior to reverse transcription would be required for distinguishing the contribution of large T gene DNA to the presumed transcript levels.

In conclusion, we have demonstrated retrospective detection of BKV RNA from routinely processed, formalin fixed, paraffin-embedded renal transplant biopsies prior to the development of histological changes. Further study in a larger population, in conjunction with contemporary diagnostic tests, may yield further information about potential diagnostic applications of this methodology.

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Conflict of interest statement. None declared.

Supplementary data

Supplementary data are available online at http://ndt.oxfordjournals.org.

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