A Rolling Circle Amplification Screen for Polyomaviruses Other than BKPyV in Renal Transplant Recipients Confirms High Prevalence of Urinary JCPyV Shedding

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Key Words

Human polyomavirus (HPyV) · JC polyomavirus (JCPyV) · Kidney transplantation · Urinary shedding · Rolling circle amplification

Abstract

Objectives: Multiple novel human polyomaviruses (HPyVs) have been discovered in the last few years. These or other, unknown, nephrotropic HPyVs may potentially be shed in urine. **Methods:** To search for known and unknown HPyVs we investigated BKPyV-negative urine samples from 105 renal transplant recipients (RTR) by rolling circle amplification (RCA) analysis and quantitative JCPyV PCR. Clinical data was analysed to identify risk factors for urinary polyomavirus shedding. **Results:** In 10% (11/105) of the urine samples RCA with subsequent sequencing revealed JCPyV, but no other HPyV sequences. Using quantitative JCPyV PCR, 24% (25/105) of the samples tested positive. Overall sensitivities of RCA of 44% (11/25) in detecting JCPyV in JCPyV DNA-positive urine and 67% (10/15) for samples with JCPyV loads >10,000 copies/ml can be assumed. Despite frequent detectable urinary shedding of JCPyV in our cohort, this could not be correlated with clinical risk factors. **Conclusion:** Routine urinary JCPyV monitoring in BKPyV-negative RTR without suspected poly-

omavirus-associated nephropathy might be of limited diagnostic value. As RCA works in a sequence-independent manner, detection of novel and known polyomaviruses shed in sufficient quantities is feasible. High-level shedding of HPyVs other than BKPyV or JCPyV in the urine of RTR is unlikely to occur.

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Introduction

Polyomaviruses are small non-enveloped viruses with circular double-stranded DNA genomes approximately 5 kb in length [1]. Besides the well-characterized human polyomaviruses (HPyVs) BK virus (BKPyV) and JC virus (JCPyV), numerous ‘novel’ HPyVs have been discovered in recent years [reviewed by 2, 3]: the Merkel cell polyomavirus (MCPyV) [4], WUPyV [5], KIPyV [6], TSPyV [7], HPyV-6, -7 [8], -9 [9], -10 [10] (MWPyV [11]/MXPyV [12]), -12 [13], STLPyV [14] and NJPyV [15].

Many studies have investigated the role of BKPyV [16] in causing polyomavirus-associated nephropathy

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(PVAN) in renal transplant recipients (RTR) [17]. Its diagnosis is based on the detection of polyomavirus in the transplant biopsy, e.g. via immunohistochemistry and also by quantitative PCR monitoring of the BKPyV load in urine and peripheral blood. Very high BKPyV urine loads and detection of high BKPyV DNAemia levels (i.e. >10,000 copies/ml) are indicators of a BKPyV-associated nephropathy [18–21]. JCPyV, the causative agent of progressive multifocal leukoencephalopathy [22, 23], is also frequently shed in the urine of both RTR and immunocompetent individuals, but only a few cases of PVAN have been attributed to JCPyV [24–27].

HPyVs are mainly transmitted during childhood – or might be of donor origin in RTR [28] – and establish persistent infections that can reactivate upon immunosuppression of the host [29]. Seroprevalence data show the presence of antibodies against a broad range of HPyV in healthy adults/blood donors (BKPyV 82%, JCPyV 39–51%, KIPyV 55–67%, WUPyV 69–89%, MCPyV 25–69%, HPyV-6 69%, HPyV-7 35% and HPyV-9 47%, TSPyV up to 70%) and organ transplant recipients (BKPyV up to 97%, JCPyV up to 80% and HPyV-9 41–63%) [8, 30–35].

Due to the increasing spectrum of recently identified polyomaviruses and their ability to establish persistent infections one might expect shedding of additional, potentially unknown HPyVs in the urine, in particular in immunocompromised patients. In the past, novel HPyVs such as TSPyV, HPyV6 and HPyV7 and also animal polyomaviruses have been successfully identified using the rolling circle amplification (RCA) technique [7, 8, 36–38], which employs a phi29 polymerase to amplify circular DNA in a sequence-independent manner [39].

The aim of this study was to identify known or unknown nephrotropic polyomaviruses in urine from 105 BKPyV-negative RTR using an RCA screen. In addition, we analysed clinical data and histopathological findings from renal biopsies to identify potential risk factors for polyomaviruria.

Materials and Methods

Patients, Specimens and Study Design

One hundred and five RTR (median age 51 years, range 18–72 years, 68% male; table 1), who underwent monitoring for BKPyV-associated nephropathy at the outpatient clinic of the Department of Nephrology, Hannover Medical School, between 02/2010 and 12/2011 participated in this study. Patients' written informed consent was obtained and the study was approved by the local ethics committee of Hannover Medical School (No. 1425-2012). Patients were included if they had undergone kidney transplantation in the previous 14 months and if the current urine sample obtained for

Table 1. Patient characteristics and virological findings of the 105 RTR (renal transplant recipients)

Patients, n	105
Gender male, n (%)	71/105 (68)
Median age at transplantation, years (range)	51 (18–72)
Immunosuppressive regimen, n (%)	
Corticosteroids	105 (100)
Antimetabolites	98 (93)
CNI	101 (96)
m-TOR inhibitors	5 (5)
Median time of urine sampling after transplantation, days (range)	180 (6–387)
Positive JCPyV PCR from urine, n (%)	25/105 (24)
Positive RCA result, n (%)	11/105 (10)
Positive anti-SV40 immunohistochemistry ^a , n (%)	1/88 (1)

^a Data available from 88 patients.

routine diagnostic BKPyV load monitoring produced a negative result following BKPyV PCR (Real-Star BKPyV PCR Kit, Altona GmbH; limit of detection 3.6×10^2 copies/ml).

The urine samples were obtained at a median of day 180 (range 6–387) after kidney transplantation and were tested using RCA and quantitative JCPyV PCR. In addition, JCPyV PCR was performed on peripheral EDTA blood samples (also obtained for routine BKPyV monitoring on the same day as the urine samples) for all patients who were found to be positive following JCPyV PCR from urine. Clinical data such as renal function or immunosuppressive regimen and – if available – histopathological biopsy results (acute rejection or presence of PVAN) were extracted from medical records to identify factors potentially associated with urinary HPyV shedding. Renal function was assessed using serum creatinine levels (range of normal values: 59–104 µmol/l for male and 45–84 µmol/l for female patients, respectively) and the estimated glomerular filtration rate (eGFR, calculated according to the Chronic Kidney Disease Epidemiology Collaboration group [40, 41]; normal value >90 ml/min/1.73 m²).

Histopathology

Histopathology results from renal biopsies (protocol or indication biopsies according to the Hannover protocol biopsy programme [42]) were available for 88/105 patients. Biopsies were taken on the same day (n = 82) or within 6 weeks before or after (n = 6) the tested urine sample was obtained. All transplant renal biopsies were diagnosed by a nephropathologist (J.U.B., V.B.) according to the Banff classification [43] and routinely analysed for the presence of polyomavirus using Anti-SV-40 immunohistochemical (IHC) staining. A distinct tubular epithelial nuclear staining was regarded as positive and qualified for a diagnosis of PVAN.

DNA Isolation and PCR

Total DNA from 200 µl of urine or EDTA whole blood was isolated using the QIAamp Blood Mini Kit (Qiagen) according to the manufacturer's recommendations and eluted in a volume of 50 µl. Samples were stored at 4° for a maximum of 24 h until DNA extraction; extracted nucleic acids were stored at –20°. Quantitative real-time JCPyV PCR (JCV Q-PCR Alert Kit, Nanogen Advanced Diag-

nostics S.r.L.; limit of detection 2.5×10^2 copies/ml; linear quantification between 2.5×10^2 and 2.5×10^7 copies/ml) was performed on 20 μ l of the extracted DNA on an ABI 7500 (Applied Biosystems).

RCA, Restriction Analysis, Cloning and Sequencing

Rolling circle amplification (RCA) was performed using the REPLI-g Kit (Qiagen) as recommended by the manufacturer using 5 μ l of isolated DNA; 5 μ l of the resulting RCA product was digested with 10 units of restriction enzyme and screened via gel electrophoresis. Following in silico restriction analysis of polyomavirus reference strains we chose the enzymes *Xba*I, *Bam*HI or *Eco*RI, which were likely to cut all currently known human polyomavirus reference genomes once (JCPyV, BKPyV, STLPyV, MWPyV, MCPyV, WUPyV, KIPyV, TSPPyV, NJPyV) or twice (HPyV-6, -7, -9 and -12), and thus were likely to lead to distinct restriction patterns upon gel electrophoresis. In addition, the restriction assays using these enzymes would also recognize 33 out of 36 animal reference polyomavirus genomes (23/33 with a single cutting site, 10/33 with two cutting sites).

If a fragment pattern corresponding to the size of a linearised polyomavirus genome was observed, the rest of the RCA reaction was digested with 60 units of the appropriate restriction enzyme, fragments were extracted and cloned into the pBluescript KS II + vector. The constructs were sequenced using M13 primers (forward GTA AAACGACGGCCAGT, reverse GGAAACAGCTATGACCATG) on an ABI 3130 sequencer (Applied Biosystems) and sequences analysed using BLAST to screen the nucleotide collection database.

Statistics

Statistical analysis was performed using SPSS version 19.0 (SPSS Inc.); *p* values <0.05 were considered significant in two-sided tests. Group comparisons were performed using Pearson's χ^2 or Fisher's exact test where appropriate for categorical variables, e.g. presence of rejection, gender, changes in the eGFR or qualitative test results. The Mann-Whitney U test was applied to determine differences between groups relating to quantitative parameters, such as age, serum creatinine or viral load. For JCPyV load values above or below the limit of linear quantification by the JCPyV PCR, statistical calculations were performed using the respective limits as surrogate values, i.e. 2.5×10^2 copies/ml for values below the PCR limit of linear quantification and 2.5×10^7 copies/ml for values above the limit of linear quantification. To statistically calculate changes of the graft function we divided patients into three subgroups upon follow-up: decrease in the eGFR of 10% or more, eGFR changes of less than 10%, and increase in the eGFR of 10% or more than compared to the initial eGFR, respectively.

Results

Detection of the HPyVs by RCA

Using the RCA screening assay with restriction enzyme analysis and subsequent Sanger sequencing 11/105 (10.4%) urine samples tested positive for JCPyV (table 1). In 6 other samples cloning and sequencing of fragments revealed sequences derived from either human DNA (*n* = 1) or TT viruses (*n* = 5). No other polyomaviruses except JCPyV were detected.

Fig. 1. JCPyV loads in urine samples that tested negative or positive by RCA. JCPyV DNA was detectable by RCA in 11 out of 25 urine samples that tested positive by quantitative JCPyV PCR. The box plot shows that RCA-positive urine samples had a significantly higher median JCPyV load (4.4×10^6 copies/ml; Mann-Whitney U test: *p* <0.001) than samples tested negative by RCA (median 1.1×10^3 copies/ml).

JCPyV Real-Time PCR in Urine or Peripheral Blood

In total 25 out of 105 (23.8%) urine samples were positive upon quantitative JCPyV PCR (table 1; median viral load 1.2×10^4 copies/ml); 4 samples were below the limit of linear quantification (2.5×10^2 copies/ml) and 5 above the limit of linear quantification (2.5×10^7 copies/ml). For all patients with JCPyV DNA-positive urine samples, peripheral blood was screened for JCPyV DNA using quantitative PCR on EDTA blood samples obtained on the same day as the urine samples. Only 3 out of the 25 blood samples were faintly JCPyV DNA-positive ($<2.5 \times 10^2$ copies/ml). JCPyV loads from the urine of the respective patients were relatively high (4.4×10^6 copies/ml in 1 case and $>2.5 \times 10^7$ copies/ml in the 2 other patients). The median urinary JCPyV load of these patients was statistically significantly higher compared to the urinary JCPyV load of patients without JCPyV DNAemia (*p* = 0.023).

Sensitivity of the RCA Determined by Quantitative JCPyV Real-Time PCR

All 11 urine samples which were positive upon RCA also tested positive by quantitative JCPyV PCR. The RCA-positive samples had a significantly (*p* <0.001) higher median urinary JCPyV load (4.4×10^6 copies/ml, range 3.8×10^3 to $>2.5 \times 10^7$) than RCA-negative samples, which had a median of 1.1×10^3 (range $<2.5 \times 10^2$ to 5.3×10^5) copies/ml (fig. 1). RCA yielded a positive result in only 1 out of 10 samples with JCPyV

Table 2. Comparison of clinical results from patients with and without positive JCPyV DNA detection in urine

	JCPyV DNA negative in urine (n = 80)	JCPyV DNA positive in urine (n = 25)
Gender male, n (%)	56/80 (70)	15/25 (60)
Median age at transplantation, years (range)	52 (18–71)	43 (18–72)
Immunosuppressive regimen, n (%)		
Corticosteroids, CNI and antimetabolite	73/80 (91)	21/25 (84)
Corticosteroids plus antimetabolite or CNI	3/80 (4)	3/25 (12)
Corticosteroids plus antimetabolite or CNI plus m-TOR inhibitor	4/80 (5)	1/25 (4)
Mean serum creatinine levels on day of urine sampling, $\mu\text{mol/l}$ (range)	158 (79–419)	160 (96–287)
Mean eGFR on day of urine sampling, ml/min/1.73 m^2 (range)	45.9 (13.9–91.3)	45.5 (19.5–86.7)
Mean serum creatinine levels on day of follow-up, $\mu\text{mol/l}$ (range) ^a	153 (84–386)	148 (91–269)
Mean eGFR on day of follow-up, ml/min/1.73 m^2 (range) ^a	49.5 (13.9–99.0)	50.4 (18.5–85.0)
Changes in eGFR upon follow-up, n (%) ^a		
Decrease of 10% or more	19/77 (25)	3/25 (12)
Less than 10% change	21/77 (27)	11/25 (44)
Increase of 10% or more	37/77 (48)	11/25 (44)
Acute rejection ^b , n (%)	16/68 (24)	5/20 (25)
Borderline	11/68 (16)	2/20 (10)
Banff IA and B	2/68 (3)	1/20 (5)
Banff IIA and B	3/68 (4)	2/20 (10)

None of the presented parameters showed statistically significant differences between patients with and without urinary JCPyV shedding. ^a Data available for 102 patients. ^b Data available for 88 patients.

loads $\leq 1.0 \times 10^4$ copies/ml, but in 10 out of the 15 samples with JCPyV loads $> 1.0 \times 10^4$ copies/ml. As the RCA method works in a sequence-independent manner these results for JCPyV can be used to roughly calculate the sensitivity of RCA for the detection of polyomaviruses in general: using our protocol a sensitivity of the RCA assay of 44% (11 out of 25) for HPyV DNA-positive urine samples in general and of 66.6% (10 out of 15) for samples with HPyV loads $> 10,000$ copies/ml can be assumed.

Histopathology Results

For 88 patients renal biopsy results were available, one of which showed positive nuclear polyomavirus staining but without associated interstitial inflammation or tubulitis (day 180 after transplantation). This patient also tested positive for JCPyV by RCA and had a high JCPyV load upon quantitative PCR ($> 2.5 \times 10^7$ copies/ml) from a urine sample obtained on the same day as the biopsy. However, in a follow-up biopsy (day 305 after transplantation) the polyomavirus immunohistochemistry was negative and did not confirm the diagnosis of JCPyV-associated nephropathy. Four EDTA blood samples obtained between day 46 and day 305 after transplantation tested negative for JCPyV DNA al-

though the corresponding urine samples contained more than 2.5×10^7 JCPyV genome copies/ml.

Twenty-one (23.9%) out of 88 patients for whom a renal biopsy was available showed signs of acute rejection (borderline n = 13; Banff IA + IB n = 3; Banff IIA + IIB n = 5). Five out of these 21 patients had positive JCPyV PCR results from urine (table 2; range of JCPyV loads $< 2.5 \times 10^2$ to $> 2.5 \times 10^7$ copies/ml) and 1 patient simultaneously had a low positive JCPyV PCR result from blood.

Clinical Data

On the day of urine sampling the patients' mean serum creatinine level was $158 \mu\text{mol/l}$ (range 79–419) and the mean eGFR was $45.7 \text{ ml/min/1.73 m}^2$ (range 13.9–91.3). For 102/105 patients follow-up data for serum creatinine levels (mean 152, range 84–386 $\mu\text{mol/l}$) and the eGFR (mean 49.1, range 13.9–99.0 ml/min/1.73 m^2) were available at a median of day 466 (range 75–917) after the initial measurement. Upon follow-up, 22 out of the 102 patients showed a decrease in eGFR of 10% or more, 32 patients of less than 10%, and 48 patients an increase in eGFR of more than 10% compared to the initial eGFR. Ninety-four of 105 (89.5%) patients received a combination of corticosteroids, a calcineurin inhibitor (CNI; cyclosporine or tacrolimus) and an antimetabolite (mycopheno-

late mofetil or azathioprine) as an immunosuppressive treatment. Six (5.7%) other patients received corticosteroids plus an antimetabolite or CNi and 5 patients (4.8%) received corticosteroids plus an antimetabolite or CNi and additionally an m-TOR inhibitor (sirolimus or everolimus).

No statistically significant differences were detected between patients with or without JCPyV shedding in the urine with respect to gender, age, presence of acute rejection, kind of immunosuppressive regimen, serum creatinine or eGFR levels on the day of urine sampling/upon follow-up or changes in the eGFR upon follow-up (table 2). Analogously, the patient subgroup ($n = 25$) tested for JCPyV in peripheral blood showed no significant differences between patients with and without JCPyV DNAemia with respect to the parameters described above (data not shown).

Discussion

BKPyV-negative urine samples from 105 RTR were screened for nephrotropic HPyVs other than BKPyV using RCA and quantitative JCPyV PCR. With the exception of JCPyV we did not find any known or unknown HPyVs in the samples using the RCA method. These results are consistent with data from others, who either could not detect HPyV-6, -7, -9, KIPyV, WUPyV, TSPyV or MWPyV in urine samples using real-time PCR [44, 45] or reported detection only rarely and at very low concentrations (HPyV-9, KIPyV and WUPyV) [3, 9, 46, 47].

Nevertheless, the detection of JCPyV genomes by RCA in two thirds of urine samples with JCPyV loads $>1.0 \times 10^4$ copies/ml in our study indicated a sufficient sensitivity of the RCA to identify HPyVs that are shed at significant concentrations into the urine. Due to the sequence-independent nature of the RCA technique a similar sensitivity to that for JCPyV can be assumed for other polyomaviruses. According to our *in silico* restriction analysis we expect that our protocol will be able to detect the majority of known polyomaviruses (the currently known 13 HPyVs as well as a large variety of animal polyomaviruses). In addition, sequencing of RCA products revealed TT virus sequences in some cases. TT viruses are known to be shed in various body fluids and have a circular genome that is preferentially amplified by the phi29 polymerase of the RCA assay [48–50]. Thus, we presume that the detection of potential novel, unknown HPyVs is also feasible using the RCA method. As a potential limita-

tion of this study, the preselection of BKPyV-negative urine samples might have influenced the likelihood of finding novel HPyVs in the urine: BKPyV-negative patients might have a better graft function and less immunosuppression, thus, they might be less prone to polyomavirus shedding.

Our cohort showed a prevalence of 24% for JCPyV DNA in the urine. This is within the range of urinary JCPyV shedding prevalence (3–49%) reported for RTR [24, 51–54]. Only one of our patients with detectable JCPyV from the urine was initially suspected to have JCPyV-associated nephropathy due to a positive immunohistochemical staining in one of his renal biopsies, but he did not develop full-blown PVAN and all follow-up diagnostics remained negative. Neither acute graft rejection nor particular immunosuppressive treatment regimens were related to urinary JCPyV shedding in our cohort. Similarly, others reported that the immunosuppressive regimen does not affect the development of urinary JCPyV shedding and even found a lower acute graft rejection rate in patients with urinary JCPyV shedding versus patients without [55, 56]. In another study a lower mean creatinine clearance in solid organ recipients with urinary JCPyV shedding compared to BKPyV or no HPyV shedders was observed [57]. In contrast, we did not find significant differences in graft function with respect to serum creatinine levels or eGFR between patients with or without urinary JCPyV shedding. These findings, together with the high prevalence of urinary JCPyV shedding, could indicate that routine monitoring of JCPyV in the urine of BKPyV-negative RTR without suspected PVAN might be of limited diagnostic value. However, data relating to the clinical significance of JCPyV replication in RTR are scarce, and thus more intensive, prospective studies are necessary.

In conclusion, although we did not discover novel HPyVs in this cohort of 105 BKV-negative RTR, the RCA technique was successfully used for the sequence-independent amplification and identification of polyomaviruses in the urine. A high prevalence of known or unknown HPyVs other than BKPyV and JCPyV shed in clinically relevant concentrations (e.g. $>1.0 \times 10^4$ copies/ml) in the urine is therefore unlikely.

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