Importance of molecular diagnostic of viral infections in renal transplant recipients

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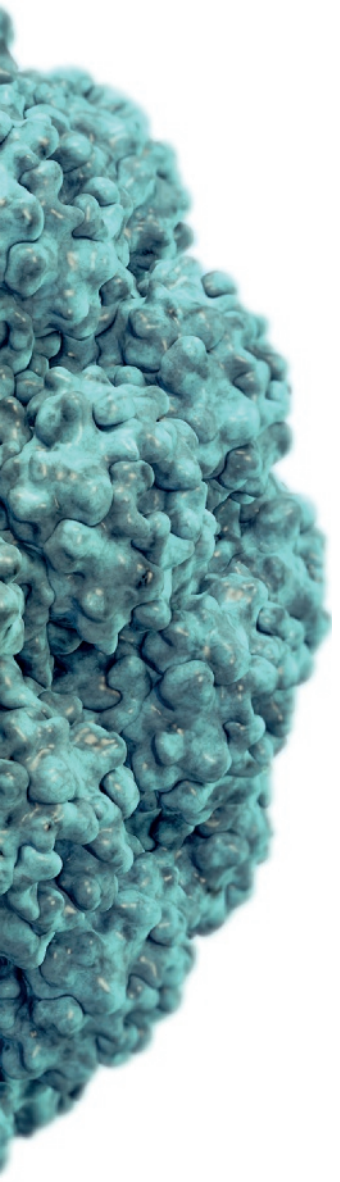

CHAPTER 3

Longitudinal monitoring of BKPyV miRNA levels in renal transplant recipients with BKPyV related pathology reflects viral DNA levels and remain high in viremia patients after clearance of viral DNA.

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ABSTRACT

Background

It is unclear whether polyomavirus BK (BKPyV) miRNA measurement has additional diagnostic and predictive value in renal transplant recipients (RTR) as compared to current methods of monitoring BKPyV DNA loads.

Methods

A retrospective, longitudinal study was performed in 30 RTR with BKPyV viruria (n = 10), BKPyV viremia (n = 10) or BKPyVAN (n = 10). Bkv-miR-B1-3p and 5p and BKPyV DNA-load were measured in urine and plasma and compared using receiver operating characteristic (ROC) curves.

Results

Levels of Bkv-miR-B1-3p and 5p and BKPyV DNA correlated strongly. Overall mostly analogue courses of urinary and plasma miRNA and DNA loads were observed. Areas under the ROC curves were not significantly different between miRNAs and DNA. Only, in contrast to BKPyV DNA load BKPyV miRNA levels increased from 6 to 12 months in the viremia group, while in the BKPyVAN group a comparable decline was seen in both DNA as miRNA.

Conclusions

In study we could not demonstrate an additional value of BKPyV miRNA detection compared to BKPyV DNA monitoring in the early post renal transplant phase. We did observe significant differences between the viremia and the BKPyVAN groups during follow up. Future studies are necessary to elucidate underlying mechanisms and clinical value of these data.

INTRODUCTION

MicroRNA's (miRNA's) are small 20-22 nucleotide long RNAs that post-transcriptionally down regulate protein levels by binding to mRNAs and block translation or induce degradation [1]. Viral miRNAs often bind with an exact complementary host mRNA target, or to viral mRNA, resulting in immune evasion mechanisms that enable latent survival of the virus in infected host cells [2,3].

With new, more potent immunosuppressive medication in renal transplant recipients (RTR), problems such as BKPyV replication have emerged [4,5].The BKPyV genome consists of a double stranded DNA molecule with a non-coding control region (NCCR), which codes for proteins active in early infection and replication (large T antigen, small T antigen and truncated antigen) and proteins active in late infection (mainly structural proteins VP1, VP2 and VP3). The late transcripts also encode for a precursor miRNA, which is cleaved into two functional miRNAs, Bkv-miR-B1-3 prime (3p) and Bkv-miR-B1-5p miRNA. These miRNAs are complementary to a segment of the large T antigen (Tag), and binding of the miRNA to the Tag transcript results in a posttranscriptional down regulation of this viral protein [3]. Besides the regulatory effect on translation of this viral protein, Bkv-miR-B1-3p also targets the human ULBP3 mRNA. ULBP3 is a ligand for the natural killer cell activating receptor NKG2D. Loss of ULBP3 leads to inhibition NK cell activation and thereby leads to reduced cytotoxicity of these cells. [6,7].

Results of recent clinical studies in RTR with BKPyV related pathology showed that BKPyV miRNAs can be detected in multiple body fluids, including cerebrospinal fluid, plasma and urine [8]. Furthermore, Bkv-miR-B1-3p and 5p can be detected in plasma and urine of patients with BKPyV virus associated nephropathy (BKPyVAN). This indicates that these miRNAs might be used as a diagnostic marker or a therapeutic target [8,9].

So far little is known about the expression of the Bkv-miR-B1-3p and 5p from early latent infection to BKPyVAN. Also, no data are available about the prognostic value of BKPyV miRNA levels, compared to the standardly used BKPyV DNA measurements. This requires a longitudinal study approach with consecutive measurements. In this study we measured BKPyV DNA and Bkv-miR-B1-3p and 5p in a longitudinal manner in patients with either BKPyV viruria, viremia or BKPyVAN.

MATERIAL AND METHODS

Patients

From October 2009 until February 2014, 30 patients, who underwent a first or second renal transplant at the University Medical Center Groningen (UMCG), were selected from a cohort of in total 811 RTR, according to their BKPyV virus infection status. This group of 30 patients consisted of 10 patients with isolated BKPyV viruria, 10 patients with BKPyV viremia and 10 patients with biopsy proven BKPyVAN.

Immunosuppression consisted of quadruple therapy with basiliximab induction, prednisolone together with calcineurin inhibition with either tacrolimus (Tac) or cyclosporine A (CsA) and mycophenolate mofetil (MMF) or mycophenolate sodium (MPS) as an antimetabolite. According to protocol RTR received CsA before 2012 and from 2012 onwards Tac.

All patients received a protocol renal biopsy at 12 months or on medical indication. Biopsy-proven T-cell mediated rejection was treated with methylprednisolone pulses. Refractory rejection episodes were treated with rabbit antithymocyte globulin (5 doses 2.5 mg/kg rATG: Merieux).

BKPyV DNA load was measured in urine and plasma samples collected at outpatient hospital visits. Selected time points for analysis were: 4 weeks prior of BKPyV replication in urine, first day of BKPyV replication in urine, 2, 4, 6 and 12 months after observed BKPyV replication. Viruria and viremia were defined as BKPyV DNA loads above log 2 cp/ml in urine and plasma, respectively. Protocol biopsies and biopsies performed under suspicion of BKPyV virus nephropathy, were stained for simian virus 40 (SV40) large T antigen. Histological proven BKPyV nephropathy was defined as interstitial inflammation and tubulitis in combination with a positive SV40 nuclear staining in tubular epithelial cells.

The Ethics Committee of the UMCG decided that the study did not fall under the scope of the Medical Research Involving Human Subjects Act (METc 2015/448).

BKPyV DNA and miRNA reverse transcriptase quantitative PCR

BKPyV DNA was measured with an internal controlled quantitative in-house quantitative PCR (qPCR), amplifying 131 bp on the VP2, with a detection limit of log 2 cp/ml as previously described by Gard et al. [10]. Briefly, samples were extracted according to the manufactures instructions, using 190 µl sample with the addition of 10 µl seal herpes virus (PhHV), as internal control [11]. Primer sequences were described by Gard et al. [10] All PCR reactions were performed with 20µl DNA and 30 µl PCR mix, containing 2x Universal Mastermix (Thermofisher, USA), 5mg/

ml Bovine plasma albumin (Roche Diagnostics, Germany), 300nM primers and 100nM probes and DNAse/RNAse free water (Sigma, The Netherlands). The ABI PRISM 7500 (Life Technologies, USA) was used for the detection and amplification using the thermal profile: 50°C for 2min, 95°C for 10min followed by 42 cycles of 95°C for 15sec, 60°C for 1 min.

Small RNAs were isolated using Favorgen miRNA isolation kit (Favorgen Biotech Corporation, Taiwan). Reverse transcription was performed using Applied Biosystems miRNA reverse transcription kit. Reverse transcription mix consisted of 1.5 µl reverse transcription buffer (10x), 1 µl RT Bkv-miR-B1-3p primer, 1 µl Bkv-miR-B1-5p primer, 0,15 µl 100mM dNTP's, 1 µl 5 U/l multiscribe Reverse Transcriptase 0,19 µl 20 U/l RNase inhibitor, 5 µl RNA isolation product and 5,16 µl RNase-free water. Incubation consisted of 30 minutes at 16°C, 30 minutes at 42°C and 5 minutes at 85°C.

Amplification was performed using Taqman miRNA assays (Applied Biosystems, Foster City, CA) with Taqman BK virus specific miRNA primers (assay IDs: bkv-miR-B1-5p:007796, bkv-miR-B1-3p:006801, Applied Biosystems, Foster City, CA). PCR reaction mix consisted of 10 µl TaqMan Universal PCR Master Mix II (2) with no UNG, 7.67 µl Nuclease-free water, 1 µl TaqMan Small RNA Assay primers and 1.33 µl RT reaction product. Samples were incubated at 95°C for 10 minutes and subsequently incubated for 40 cycli for 15 seconds at 95°C, 60 seconds at 60°C. In each reaction a positive control samples and a sample containing RNA free water were used as internal positive control and negative control, respectively.

Statistics

Statistical analysis was performed using IBM SPSS Statistics 22. Baseline characteristics were compared using Chi-square test and ANOVA for categorical variables and continuous variables, respectively. Longitudinal data were analysed using generalised estimating equations (GEE) with an exchangeable correlation matrix. Estimated marginal means (EMM) with 95% confidence intervals from the GEE analyses were plotted in graphs. Receiver operating characteristic (ROC) curves for genomic viral DNA and Bkv-miR-B1-3p and 5p RT-PCR were constructed and C-statistics (area under the curve of ROC curves) were calculated in SPSS using BKPyV virus nephropathy as state variable. Equality of the C statistic was tested via the method described by DeLong et al.[12]. Figures were plotted using Graphpad Prism 5.01. Two-sided P-values <0.05 were considered significant.

RESULTS

Baseline characteristics

Baseline characteristics of the patients are displayed in Table 1. The number of deceased donors and the cold ischemia time were significantly higher in the viremia and BKPyVAN group compared to the viruria group. Other baseline characteristics did not differ significantly between the groups (Table 1).

Table 1: Baseline characteristics of renal transplant recipients and donors

	BKPyV viruria[a] (N = 10)	BKPyV viremia[b] (N = 10)	BKPyVAN[c] (N = 10)	P value
Male N (%)	7 (70.0)	5 (50.0)	9 (81.8)	0.29
Age, years ± S.D.	49.5 ± 13.8	59.2 ± 8.9	59.7 ± 8.1	0.06
Caucasian n (%)	9 (90.0)	8 (80.0)	9 (81.8)	0.81
Primary disease leading to end stage renal failure, n (%)				0.26
Polycystic kidney disease	4 (40.0)	1 (10.0)	0 (0)	
Primary glomerular disease/ glomerulonephritis	2 (20.0)	2 (20.0)	4 (36.4)	
Renovascular disease/ hypertension	1 (10.0)	2 (20.0)	2 (18.2)	
Diabetes Mellitus	0 (0.0)	1 (10.0)	1 (9.1)	
Pyelonephritis or interstitial nephritis	1 (10.0)	0 (0)	0 (0)	
Urologic	0 (0)	2 (20.0)	0 (0)	
Other	2 (20.0)	2 (20.0)	4 (36.4)	
HLA AB mismatch, mean ± S.D.	1.90 ± 1.45	2.10 ± 1.45	2.64 ± 1.36	0.48
HLA DR mismatch, mean ± S.D.	0.90 ± 0.74	0.90 ± 0.74	1.18 ± 0.75	0.61
Number of kidney transplantation absolute numbers n (%)				0.34
1	10 (100.0)	9 (90.0)	11 (100.0)	
2	0 (0)	1 (10.0)	0 (0)	
Donor characteristics				
Age, years ± S.D.	49.1 ± 10.7	53.1 ± 16.7	53.9 ± 14.3	0.73
Type of transplantation (%)				0.001
Living	10 (100.0)	2 (20.0)	4 (36.4)	
Deceased	0 (0)	8 (80.0)	7 (63.6)	
Cold ischemia time, deceased donors only, hours ± S.D.	0.6 ± 0.2	10.3 ± 7.4	7.1 ± 6.3	0.001

a: Polyomavirus BK viruria, b: Polyomavirus BK viremia, c: Polyomavirus BK associated nephropathy.

Immunotapering and renal biopsies

All patients in the BKPyVAN group received a diagnostic renal biopsy on average 67 days after BKPyV replication. Immunosuppression was tapered in all patients on average 72 days after observed BKPyV replication.

In the viremia group 4 out of 10 patients received a renal biopsy under suspicion of BKPyVAN at on average 111 days after BKPyV replication, which was not detected in the biopsies. All patients, including the 6 patients not under suspicion of BKPyVAN in the viremia group, received a protocol biopsy at 12 months in which no BKPyVAN was detected. Immunosuppression was tapered in 7 patients, including the 4 patients under suspicion of BKPyVAN, on average 65 days after observed BKPyV virus replication.

Primary outcomes BKPyV viruria, viremia, and BKPyVAN

At 329 of 360 time points (91,4%) samples were collected and analyzed, with an average number of measured time points per patient of 5,5. When miRNA Ct values from BKPyV viruria, viremia and BKPyVAN patients were correlated to BKPyV DNA loads, a significant correlation was found for Bkv-miR-B1-3p and BKPyV DNA (Pearson R: -0.87, $p < 0.0001$) and for Bkv-miR-B1-5p and BKPyV DNA (Pearson R: -0.76, $p < 0.0001$) (Figure1).

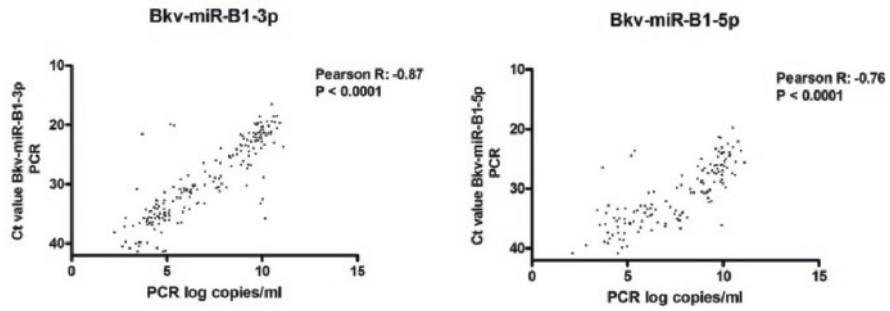

Figure 1: Correlation of BKPyV virus DNA with Bkv-miR-B1-3p and Bkv-miR-B1-5p.
Correlation between BKPyV miRNA and DNA load measured across the three research groups from 0 to 12 months after observed BKPyV replication. Correlation between Bkv-miR-B1-3p and BKPyV (A). Correlation between Bkv-miR-B1-5p and BKPyV (B).P values were calculated using Pearson's correlation coefficient.

Longitudinal analysis

Urine

A different course of urinary Bkv-miR-B1-3p and 5p EMM was found in the viruria group compared to the viremia and the BKPyVAN group. In the viruria group a peak EMM of Ct-values 31 and 35 at 2 months after BKPyV replication was found for Bkv-miR-B1-3p and 5p, respectively.

These Ct-values declined from month 2 to 12 in the viruria group. In the viremia and BKPyVAN group urinary Bkv-miR-B1-3p and 5p Ct-values showed a peak at month 4 with Bkv-miR-B1-3p Ct-values of 22 (viremia) and 23 (BKPyVAN) and Bkv-miR-B1-5p Ct-values of 27 (viremia) and 29 (BKPyVAN). Both declined from 4 to 12 months. This difference in peak value and course of replication between patients with BKPyV viruria versus BKPyV viremia and BKPyVAN was significant (viruria – viremia Bkv-miR-B1-3p p = 0.02, Bkv-miR-B1-5p p = 0.02, viruria – BKPyVAN Bkv-miR-B1-3p p = 0.02, Bkv-miR-B1-5p p = 0.003), and remained significant after adjustment for donor type, and cold ischemia time (Figure 2A and 2B).

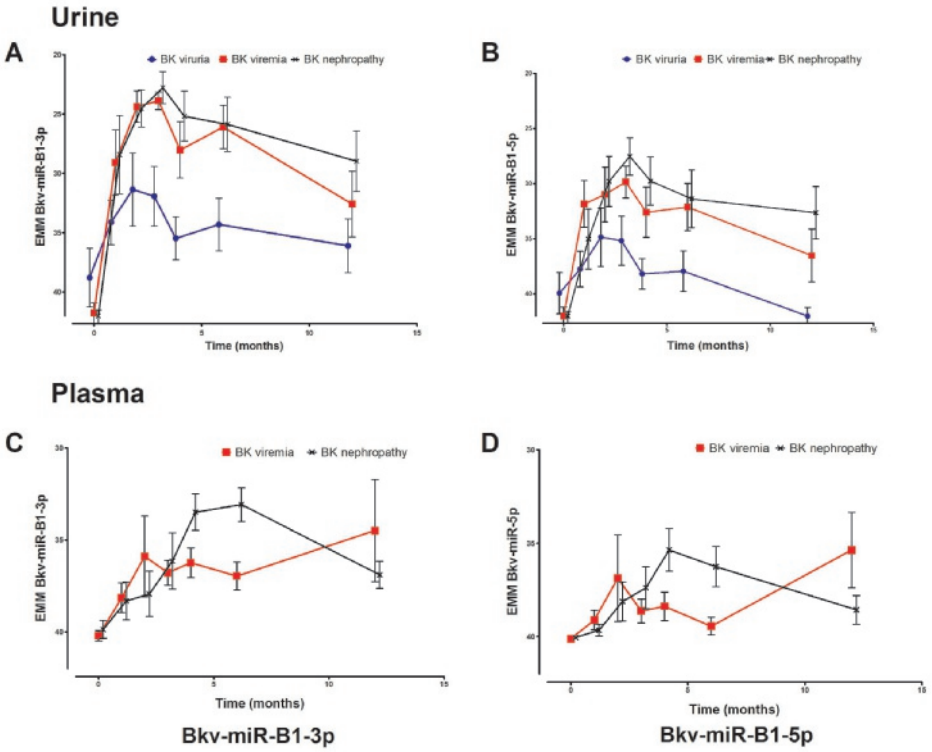

Figure 2: Longitudinal analysis of Bkv-miR-B1-3p and Bkv-miR-B1-5p.

Longitudinal course of BKPyV miRNA Ct values from t = 0 months to t = 12 months after observed BKPyV replication, by research group, viruria (blue circles), viremia (red squares) and BKPyVAN (black crosses). Estimated marginal means (EMM) of Bkv-miR-B1-3p in urine (Figure 2A) and plasma (Figure 2C). Estimated marginal means (EMM) of Bkv-miR-B1-5p in urine (Figure 2B) and plasma (Figure 2D). P values were calculated using GEE with an exchangeable correlation structure.

In the viruria group a peak in urine DNA load is seen at 2 months after observed BKPyV replication with loads up to 6 log cp/ml, declining to a final load of 5 log cp/ml at 12 months (Figure 3A). In the viremia and the BKPyVAN group mean urine DNA loads peak at 4 months after observed BKPyV replication at 10 log cp/ml and decline to a load of 7 log cp/ml. This

course is significantly different between viruria and viremia patients (p <0.0001) and viruria and BKPyVAN patients (p <0.0001) (Figure 3A). Overall courses of Bkv-miR-B1-3p and 5p strongly resemble the courses of corresponding viral DNA in urine.

Plasma

Plasma levels are overall much lower. In the viruria group plasma levels of both Bkv-miR-B1-3p and 5p were undetectable.

In the viremia group, a peak of plasma Bkv-miR-B1-3p at 3 months after observed replication with a Ct-value 36.5 was observed, while in the BKPyVAN group Bkv-miR-B1-3p Ct-values peaked between 4 and 6 months at approximately 33.0. Similar observations were made for Bkv-miR-B1-5p with a peak Ct-value of 38.4 at 3 months in the viremia group compared to a peak of 35.5 between month 4 and 6 in the BKPyVAN group. Furthermore, levels of both Bkv-miR-B1-3p and 5p rose between 6 and 12 months in the viremia group to Ct-values of 34.8 and 35.1 for Bkv-miR-B1-3p and Bkv-miR-B1-5p respectively. Contrary to the observations in the viremia group, a drop from month 6 to 12 to Ct-values of 37.1 and 38.5 for Bkv-miR-B1-3p and 5p respectively, was seen in the BKPyVAN group. These differences in miRNA levels over time between viremia and BKPyVAN RTR were significant with p values of 0.005 for in Bkv-miR-B1-3p and 0.001 for in Bkv-miR-B1-5p and remained significant after adjustment for donor type and cold ischemia time (Figure 2C and 2D).

By definition, no viral DNA was detected in plasma of patients with viruria. During the 12 months study period, patients with viremia showed an increasing viral load up to 4 log cp/ml from 0 to 2 months. Subsequently viral loads from 2 to 12 months decreased from 4 log cp/ml to 2.3 log cp/ml, corresponding with the moment of tapering of immunosuppression. All patients in this group remained under the threshold value of 4 log cp/ml. In contrast, EEM of viral loads in patients in the BKPyVAN increased to 5 log copies/ml, at 4 months and declined to 3.2 log copies/ml at 12 months. The course of genomic viral load between viremia patients and BKPyVAN patients was significantly different with a p-value of 0.001 (Figure 3B).

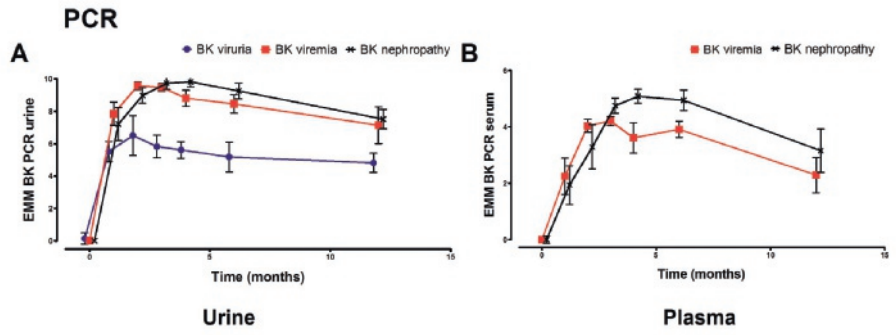

Figure 3: Longitudinal analysis of BKPyV viral load.

Longitudinal course of BKPyV DNA load (cp/ml) from t = 0 months to t = 12 months after observed BKPyV replication, by research group, viruria (blue circles), viremia (red squares) and BKPyVAN (black crosses). Estimated marginal means (EMM) of BKPyV DNA load in urine (Figure 3A) and plasma (Figure 3B). P values were calculated using GEE with an exchangeable correlation structure.

ROC curves

ROC curves of plasma Bkv-miR-B1-3p, 5p and BKPyV DNA are displayed in Figure 4. For Bkv-miR-B1-3p the optimal cut off point was at Ct-value 32.9, with a sensitivity of 83,0% and a specificity of 86,4%. Area under the curve for Bkv-miR-B1-3p was 0.86 (p <0.0001). For Bkv-miR-B1-5p the optimal cut off point was at Ct-value 35.0 with a sensitivity of 78,0% and a specificity of 86,0%. Area under the curve for Bkv-miR-B1-5p was 0.86 (p <0.0001).

The optimum cut off point for BKPyV plasma DNA load was at a viral load of 4.85 log cp/ml, which corresponded to a sensitivity of 83% and a specificity of 82%. The area under the curve was 0.89 (p < 0.0001) (Figure 4).

There was no significant difference between de AUC of Bkv-miR-B1-3p and plasma DNA load (p = 1.00), Bkv-miR-B1-5p and plasma DNA load (p = 1.00) and Bkv-miR-B1-3p and 5p (p = 1.00). No BKPyV viruria or viremia patients who did not undergo a renal biopsy had a plasma Bkv-miR-B1-3p Ct value <32.9 and a Bkv-miR-B1-5p value <35.0.

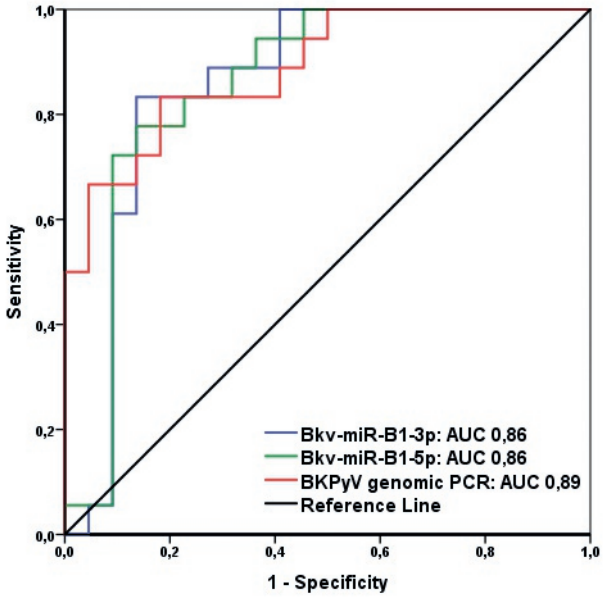

Figure 4: ROC curves of Bkv-miR-B1-3p, Bkv-miR-B1-5p and BKPyV genomic PCR.

Receiver operating characteristic (ROC) curves for Bkv-miR-B1-3p (blue line) Bkv-miR-B1-5p (green line) and genomic viral DNA (red line). C-statistics (area under the curve of ROC curves) were calculated in SPSS using BKPyV virus nephropathy as state variable.

DISCUSSION

In this study we measured Bkv-miR-B1-3p and 5p levels longitudinally in RTR. The study objective was to determine a value of viral miRNAs as a marker of BKPyVAN, compared to renal biopsy and BKPyV DNA levels. Our hypothesis was that BKPyV miRNAs would give additional information to BKPyV DNA measurement, in BKPyV virus replication and BKPyVAN.

A couple of conclusions can be drawn from this study. Firstly, a strong correlation was found between BKPyV miRNAs and BKPyV DNA levels in both urine and plasma. This correlation is also seen in longitudinal analysis of urinary miRNA, in which a higher concentration of urinary Bkv-miR-B1-3p and 5p, in viremia and BKPyVAN patients was observed, compared to viruria patients, corresponding with BKPyV DNA levels. These data show a linear association between BKPyV DNA and miRNA in time and indicate that viral miRNAs reflect the amount of virus.

Secondly, levels of Bkv-miR-B1-5p were generally lower than Bkv-miR-B1-3p levels, which makes Bkv-miR-B1-3p a more reliable biomarker than Bkv-miR-B-5p.

Thirdly, since the moment of tapering of immunosuppression was almost identical in the viremia and the BKPyVAN, the delayed increase of DNA load in RTR with BKPyVAN compared to RTR with viremia indicates a delayed respons to tapering of immunosuppression in this group.

Fourth, the increasing level of Bkv-miR-B1-3p and 5p observed during the decline of BKPyV DNA in plasma of RTR with viremia are contrary to the data of miRNA and DNA levels in the BKPyVAN group and might indicate a function for these miRNA's, in the remission phase of patients with viremia.

Importantly, in the viremia and BKPyVAN group data of the remission phase is confounded by intervention of the nephrologist: via tapering immunosuppression. Nevertheless tapering of immunosuppression seems to have taken place in all or the majority of the patients in both groups at almost the same time. Still in the viremia group BKPyV virus miRNA levels continued to rise after tapering.

Explanations of this observation are speculative. One explanation could be related to the dual function theory of BKPyV miRNAs as described by Broekema et al. and Bauman et al. [2,3,7,13]. This theory comprises of reconstitution of the non-coding control region during infection, alternating early and late promotors in this region, and thereby subsequently promoting different miRNA transcription and function of the miRNA in early and late infection. This might indicate an in vivo observation of the previously described function of BK miRNA in late

infection, the downregulation of ULBP3, a ligand for natural killer cell mediated cell lysis. It is compatible with the location of the miRNA on the late strand of the BK virus genome and is in accordance with its expression in late phase infection [3,6].

A second explanation could nevertheless be related to tapering of immunosuppression. It could be assumed that tapering of immunosuppression was done more rigorously in the BKPyVAN group compared to the viremia group. Following this hypothesis, the decline of miRNAs observed in the BKPyVAN group from 4 to 12 months post infection, could be attributed to the recovering immune defense. Recovering BKPyV virus specific T-cells might counteract the immune evasive effects of the miRNAs. In summary, compared to the BKPyVAN group, the virus might escape elimination by the immune system more effectively, in the viremia group.

The relative high Ct-values we found in plasma miRNA measurements, reaching only 33 and 35 for plasma BKPyV miRNA 3p and 5p respectively in the BKPyVAN group and even higher, hence lower levels, in the other groups, limit the effectiveness of plasma BKPyV miRNA as a marker. With these high Ct values the chance of false negative results increase. It can be questioned if plasma is the right carrier of BKPyV miRNA as a marker of disease activity. In urine, with our current method no discrimination of disease activity could be made. Future research on BKPyV miRNAs nevertheless should focus on urine as a liquid biopsy source for measuring BKPyV miRNA levels. Although differences in miRNA loads reached significance at some points, no clear clinical relevance became apparent from this study.

In summary, our data indicate that BKPyV miRNA levels correlate strongly with currently used BKPyV DNA load monitoring.. This is further supported by the overlapping ROC curves of all three markers. Urine levels are higher as compared to plasma levels, making urine a more optimal liquid biopsy source for measuring BKPyV miRNA levels. Nevertheless, in patients with persistent viremia we observed rises in both 3p and 5p miRNA levels, even after decline of BKPyV DNA. This is especially remarkable given the high correlation between DNA load and miRNA levels. Future research is required to explain these contradictory findings.

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