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Detection of BKV encoded mature MicroRNAs in kidney transplant patients: Clinical and biologic insights

Yuchen Huang[a,b], Gang Zeng[a,b], Parmjeet S. Randhawa[a,b,*]

[a] Department of Pathology, The Thomas E Starzl Transplantation Institute, University of Pittsburgh, University of Pittsburgh, Pittsburgh, PA, United States
[b] Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, United States

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ABSTRACT

Background: Polyomavirus BK (BKV) encodes two mature miRNAs that regulate the viral life cycle.
Objectives: This study investigated the autoregulatory and immunomodulatory effects of these miRNAs that have been defined in culture systems, but subject to only limited exploration in clinical samples.
Methods: BKV-miR-B1-5p, BKV-miR-BJ1-3p, BKV DNA and BKV VP-1 mRNA levels were measured in 32 paired obtained plasma & urine samples from kidney transplant patients with (a) early stage infection manifesting as viruria, and (b) later stage infections complicated by viremia.
Results: All patients showed abundant urine miRNAs (7.84E + 02-1.91E + 06 copies/ml, but plasma miRNA was below the limit of detection. There was no statistically significant difference in urinary miRNA levels between viruric and viremic patients. Median 5p miRNA load was 4–6 logs lower than the BKV genomic load. Higher miRNA levels in the urine were associated not with lower but higher urinary viral loads. BKV preferentially used the 3p miRNA for its interactions with host cell mRNAs. The mean ratio of 5p/3p in patients with viruria was 0.09, and 0.03 in patients with viremia.
Conclusions: The data suggest that immune evasion functions of BKV miRNAs over-ride the negative autoregulatory feedback effects in kidney transplant patients with active viral replication.

1. Background

Polyomavirus BK (BKV) is widely latent according to serologic studies. Immunosuppression leads to viral reactivation and asymptomatic viruria in 20–60% of patients, with progressive tissue injury and viremia in 1/3rd, and viral nephropathy (BKVN) in 1–10% of allograft kidneys [1]. The only therapeutic strategy available is to reduce the immunosuppression, which leads to resolution in ˜75% of patients with a ˜5-20% risk of acute rejection and ˜2-fold increase in graft loss [2]. An increased understanding of microRNA (mirRNA) mediated regulation of BKV replication would promote effective BKVN therapies.

The late viral region of BKV encodes a primary miRNA transcript, which is complementary to 3' end of the early transcript and targets an open reading frame [3]. Drosha and Dicer enzyme cleave the targeted transcript to produce a duplex comprised of miRNAs designated as BKV-miR-B1-5p and BKV-miR-BJ1-3p [4,5]. The sequence of BKV-miR-BJ1-3p nucleotide sequence is identical for polyomaviruses BK and JC [6,7]. Another unusual aspect of BKV biology is that both the 5' terminus guide strand and the 3' terminus passenger strands of the BKV miRNA duplex are functional. Following incorporation into the miR-Induced Silencing Complex (miRISC), both the 5p and the 3p miRNAs can bind to and cleave the viral large T antigen (LTA) mRNA and thus inhibit viral replication [8].

Thus, BKV can autoregulate the rate of replication of its own genome. The 3p miRNA has binding ability for two additional targets, namely the stress induced ligands ULBP1 and ULBP3, even though it sequence is not perfectly complementary to the RNAs targeted [9,10]. ULBP3 is a ligand of the NK cell eceptor NKG2D and miRNA directed downregulation of this protein is believed to confer an immune evasion advantage to the virus as it seeks to complete its life cycle virus infected cells.

The functions of 3p and 5p miRNAs have been defined in-vitro cell culture systems and their clinical relevance are unknown [9]. Indeed, one can envision diametrically opposite effects on viral load in kidney

Abbreviations: BKV, polyomavirus BK; BKVN, BK viral nephropathy; cDNA, complementary DNA; Ct, threshold cycle; JCV, polyomavirus JC; LTA, large T antigen; miRNA, microRNA; miRISC, miR-Induced Silencing Complex; NCCR, non-coding control region; ND, not done; NKG2D, Natural Killer Group 2D; P, plasma; PCR, polymerase chain reaction; RNA, ribonucleic acid; RT, reverse transcriptase; U, urine; ULBP, Unique Long 16 (UL-16) binding protein; VP-1, viral capsid protein 1

* Corresponding author at: E737 UPMC-Montefiore Hospital, 3459 Fifth Ave, Pittsburgh, PA 15213, United States.
E-mail address: randhawapa@upmc.edu (P.S. Randhawa).

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transplant patients with active BKV replication. Thus, cleavage of LTA would inhibit viral replication and at the same time reduce the expression of a protein that is targeted by CD8 positive T-cells for elimination of the virus. Measurement of miRNAs in human samples is necessary to assess the net effect of these two competing influences on the viral life cycle. Accordingly, we have measured as BKV-miR-B1-5p, BKV-miR-BJ1-3p, BKV DNA and BKV VP-1 mRNA levels in concurrently obtained plasma & urine samples from (a) patients at an early stage of infection manifesting only as viruria, and (b) patients at a later stage of infection which has been complicated by viremia.

2. Objectives

We investigated the autoregulatory and immunomodulatory effects of polyomavirus miRNAs that have been largely defined in culture systems, and subject to only limited exploration in clinical samples.

3. Study design

3.1. Clinical material

Whole urine and plasma samples sent to the University of Pittsburgh clinical laboratory for BKV PCR were retrieved (IRB approval #000586).

Samples were selected over a course of 1 year during which 73 plasma samples were reported to have BK viremia. A total of 16 paired urine and plasma samples representing the highest plasma viral loads were selected to explore a potential relationship between BKV miRNA and clinical viral loads. This included: (a) 8 BKV positive urine (U) samples with 8 concurrent plasma (P) samples that were BKV negative (designated as U + P- patients), and (b) 8 urine samples with BK viruria with 8 corresponding plasma samples that documented viremia (designated as U + P+).

3.2. RNA extraction, quantitation of BKV miRNAs and viral DNA load

Plasma samples (200 µL) were processed using miRNeasy Serum/Plasma Kit (Qiagen) according to the manufacturer's instructions. Urine samples, 1.5 ml, spun at 2000 rpm for 15 min were used to extract total RNA by the Trizol method. Additional technical details on miRNA quantitation are provided as supplementary data. Urine and plasma BKV DNA were measured according to a published method [11].

4. Results

4.1. Clinical parameters and assay performance

The patients studied varied in age from 17 to 75 years (median 52.5). The male to female ratio was 4:1. Samples had been collected from 49 to 1460 days following transplant (median 125). In the U + P- group of patients, urinary viral load ranged from 1.74E8+ 08 to 2.84E + 10 genomic equivalents/ml (median 1.62E + 09). By definition these patients tested negative for BKV DNA in the plasma (Table 1). In the U + P+ patient group, urine viral load was expectedly higher than the U + P- group and varied between 1E + 09 to 8.8E + 09 genomic equivalents/ml (median 4.74E + 09). The plasma viral load ranged from 4.08E + 04 to 4.94E + 05 genomic equivalents/ml (median 1.28E + 05).

The median VP-1 mRNA level was 1.13E + 09 copies/ml (range 2.90E + 06 –2.1E + 10] in the U + P- group and 6.10E + 08 copies/ml (range 1.76E + 08-3.57E + 09) in the U + P+ group. Two of six clinically indicated biopsies showed mild rejection. One biopsy qualified for the diagnosis of polyomavirus nephropathy.

MiRNA assay performance: The BKV-miR-B1-5p and BKV-miR-BJ1-3p assays could detect 22 copies per reaction and had a linear dynamic range of 2E + 01 to 2E + 07 copies. The effective sensitivity in the clinical samples was 50 copies/ml for urine and 3400 copies/ml for plasma for both the 5p and 3p assays (Fig. 1). The lower sensitivity of the plasma assays reflects the lower sample volume used (0.2 ml plasma versus 1.5 ml urine). Based on its oligonucleotide sequence, the 5p assay is expected to be BKV specific whereas the 3p assay cannot discriminate between BKV and JCV. However, we did not find any samples that were 5p negative and 3p positive. Moreover, real time PCR of JCV DNA was negative in all samples (data not shown). Therefore, all viral miRNA data in this study represents BKV and not JCV infection.

4.2. BKV miRNA in patients with viruria

In the U + P- patients, BKV-miR-B1-5p load was 1.29E + 03–6.89E + 04 (median 7.29E + 03) copies/ml, which is ˜ 5 logs lower than the BKV genomic load (Fig. 2). We speculate that is because only a small fraction of BKV genomes actively transcribe miRNA, or that assays need to be performed on exosome enriched samples to detect all the miRNA molecules excreted in the urine [12]. The discordance between the level of miRNA and viral DNA is not due to RNA degradation since miRNAs are generally considered to be quite stable. Moreover, we see the same phenomenon in renal proximal tubular epithelial cultures infected with BKV (unpublished observations). BKV-miR-BJ1-3p load varied between 7.84E + 02 - 1.91E + 06 (median 9.09E + 04) copies/ml. This was 1 log higher than the median BKV-miR-B1-5p load in these samples. The median 5p/3p ratio was 0.09 indicating that BKV preferentially uses the 3p miRNA for its interactions with host cell mRNAs.

4.3. BKV miRNA in patients with viremia

In the U + P+ group, BKV-miR-B1-5p load ranged between 1.26E + 03 - 2.98E + 04 copies/ml (median1.53E + 04). This was 5–6 logs lower than the median BKV genomic load in these patients. The BKV-miR-BJ1-3p load was 4.48E + 02 - 1.86E + 06 (median 4.89E + 05) copies/ml. This was 1 log higher than the median BKV-miR-B1-5p load in these samples.

Notably, BKV-miRNA was not detected in any plasma sample, even in those with the highest levels of viremia, despite the use of primers

Table 1
BKV DNA, mRNA and miRNA load in plasma and urine*.

		Urine + Plasma-		Urine + Plasma+	
		Median	Range	Median	Range
BKV Genomic load(/ml)	Plasma	ND	ND	1.28E + 05	4.08E + 04 - 4.94E + 05
	Urine	1.62E + 09	1.74E+08 - 2.84E + 10	4.74E + 09	1.00E + 09 - 8.80E+09
BKV miRNA load(/ml)	BKV -miR-B1-5p	7.29E + 03	1.29E + 03 - 6.89E + 04	1.53E + 04	1.26E + 03 - 2.98E + 04
	BKV-miR-BJ1-3p	9.09E + 04	7.84E + 02 -1.91E + 06	4.89E + 05	4.48E + 04 - 1.86E + 06
5 P/3P Ratio		0.09	0.005 – 1.65	0.03	0.01 - 0.07
VP1 mRNA Urine(/ml)		1.13E + 09	2.90E + 06 - 2.10E+10	6.10E + 08	1.76E + 08 - 3.57E + 09

* BKV: Polyomavirus BK; ND: not detected; VP1: Viral capsid protein.

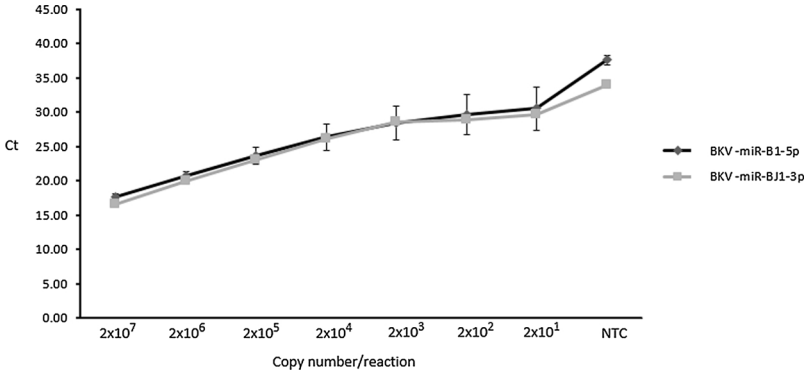

Fig. 1. Relationship between target copy number (X-axis) and detection threshold cycle (Ct, Y-axis). Ct was related inversely to the concentration of BKV-miR-B1-5p and BKV-miR-BJ1-3p synthetic oligonucleotides over a 6 log linear dynamic range (20 - 20E + 07 copies per reaction). No template control (NTC) reactions corresponded to a Ct of ~35, which was used to classify samples was BKV miRNA positive or negative. The mean CV of the Ct values obtained by us across the whole range of miRNA concentrations was 5% and 3% for the 5p and 3p assays respectively. In the clinical samples the corresponding CVs were 13% and 15% indicating that both assay variability and clinical parameters contribute to the observed data scatter.

optimized by a reputed commercial source. It is possible that miRNAs below the limit of detection were present or that assay of whole blood instead of plasma might have been more successful. Patients with viruria alone versus those with viremia showed no statistically significant difference in the urinary miRNA levels expressed as copies/ml urine. In scatter plots higher urine miR levels correlated with higher (not lower) urinary viral genomic loads (Fig. 3). Thus, the immune evasion effect of BKV miRNA induced downregulation of the NKG2D ligand ULBP3 appeared to over-ride the inhibition of viral replication that would be expected from miRNA induced cleavage of the viral large T antigen.

5. Discussion

BKV infection of the allograft kidney passes thru a series of stages, namely, activation of latent infection, viruria, viremia, and nephropathy. If miRNAs were to have a clinically meaningful autoregulatory role in controlling viral replication, one might expect that clinical samples from patients with viremia would have lower levels of BKV miRNA compared to patients who had lower levels of viruria and no viremia. However, this was not observed by us, and in fact the reverse was found. Similar relationships have been noted by others in the urine and plasma of patients with BK nephropathy [13]. Taken together, these observations suggest that the immune evasion role of BKV miRNA over-rides its effect on the downregulation of viral replication. This is likely due to two mutually complementary effects. First, high levels of 5p and 3p miRNA would cause excessive cleavage of LTA and impair the immune response mediated by CD8 + T-cells sensitized to this viral antigen [5,14]. Secondly, 3p miRNA induced downregulation of the NKG2D ligand ULBP3 would similarly compromise the antiviral role played by NK cells.

The observation that 3p miRNA was more abundant than 5p miRNA in clinical urine specimens contrasts with some prior published work. Pietila et al preferentially amplified 5p miRNAs in plasma, urine, and cerebrospinal fluid samples submitted to a virology laboratory from patients who were 'diagnosed, suspected or monitored' for severe polyomavirus associated disease [15]. Li et al also detected primarily 5p miRNA in the plasma of kidney transplant patients with BK viremia or nephropathy, but attributed this a lower sensitivity of their 3p miRNA assay [13]. The 5p assay in their study had a lower limit of detection of 1000 molecules per reaction. In a more recent study that employed urine samples from patients with systemic lupus erythematosus the 3p miRNA was the predominant form detected [16]. It was also noted that patients with biopsy proven lupus nephritis had lower levels of 5p miRNA compared to patients without lupus nephritis. The viral load in these urine specimens was much lower than our study (mean 3.3 log copies/ml) suggesting that lower levels of BKV miRNA can retain their autoregulatory function at the site of infection. The genomic configuration of the BKV non-coding control region (NCCR) may be another variable in this equation. Mutagenesis studies have shown that the archetype BKV strains can successfully regulate viral replication whereas this modulatory influence is not seen in viral strains that have a rearranged NCCR [17,18]. The NCCR architecture of the BKV strains studied by us is not known, but rearrangements in that part of the viral genome are only seen in 22% of patients with viremia [19], and 19% of patients with BKV nephropathy [20].

VP-1 mRNA levels are measured in the context of BKV infection to in an attempt to distinguish latent from lytic infection, based on the premise that mRNA expression is contingent on viral DNA replication

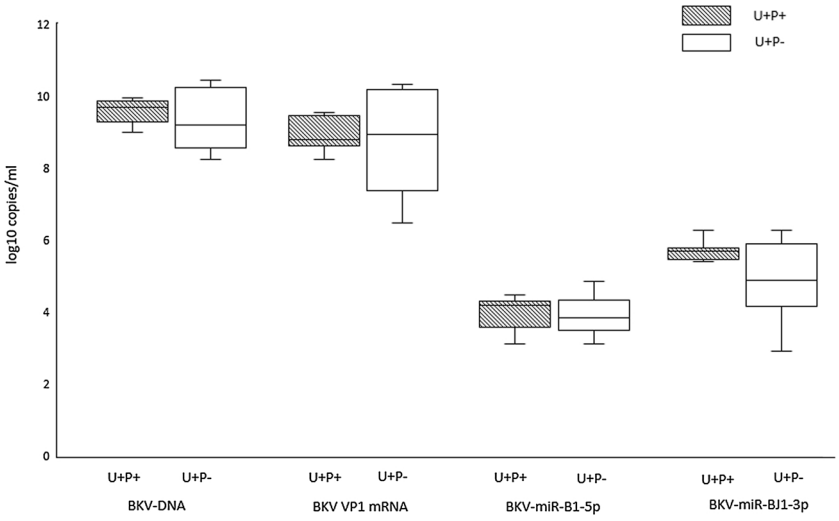

Fig. 2. BKV DNA, BKV viral capsid protein (VP-1) mRNA, BKV-miR-B1-5p and BKV-miR-BJ1-3p concentrations (median, Q1,Q3) in the urine of patients with viral DNA detected in (a) urine and plasma (U + P+) and (b) urine but not plasma (U + P-).

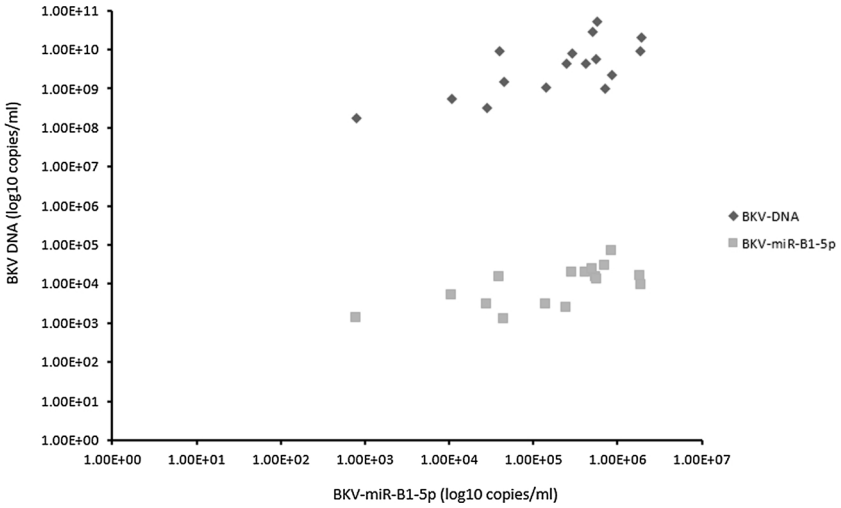

Fig. 3. BKV-miR-B1-5p (X-axis) and BKV DNA (Y-axis) concentrations (log_{10} copies/ml) in all urine samples evaluated. MiRNA copy numbers (solid squares) are several logs lower than DNA copy numbers (diamonds). Higher miRNA load correlated with higher (not lower) viral genomic load (Pearson r = 0.5).

[21]. Theoretically, one copy of the VP-1 gene can transcribe multiple copies of the corresponding mRNA. However, VP1mRNA copy numbers were in the same range as BKV DNA. This may reflect RNA degradation in our samples which were in the freezer for many years before they were assayed. Alternately, only a small percentage of infected cells were transcriptionally active.

Although, it was not the purpose of our study to evaluate the value of BKV miRNA assays in clinical diagnostics, some comments are in order as other investigators have explored this notion. miRNAs can be used to monitor BKV infection in clinical samples but requires methods that are more sensitive than DNA & mRNA assays so as to detect the substantially lower concentrations. Since BKV miRNA expression can be seen prior to viral replication the question arises whether these assays can be used to diagnose latent infection. Lagatie et al showed that JCV miRNA (but not BKV miRNA) could be found in 86% of plasma and 57% of urine specimens obtained from BKV seronegative subjects [7]. The miRNA assays used incorporated a 12 cycle preamplification step followed by an additional 45 PCR cycles. Notably, these same investigators later published a plasma miRNA profiling study of patients with JC viruria in which signals obtained > 32 cycles were regarded as noise [6]. Our own negative results in plasma sample suggest that the diagnosis of latent BKV infection based on miRNA detection would require extremely sensitive assays with careful attention to the problem of false positives. On the other hand, miRNA-based assays do offer two advantages over existing PCR assays that amplify viral DNA. First, miRNAs reflect gene expression and detection of significant amounts in the urine could be an indicator of active viral replication, as opposed to passive urinary shedding of latently infected cells. Secondly, BKV miRNA sequences are very well conserved so that a single assay can be designed to successfully diagnose all viral genotypes, minor variants and quasispecies.

In conclusion, miRNAs have potential utility to monitor active BKV infection in the urine of kidney transplant patients, although additional work is needed to ensure that the kinetics of viral miRNA and DNA correlate closely at all stages of infection. Monitoring in the plasma would require development of more sensitive assays to ensure detection of low copy numbers. Conversely, since miRNA sequences are well conserved, miRNA based diagnostic assays are not expected to be affected by known polymorphisms in the viral genes targeted in conventional DNA based PCR assays. While BKV miRNAs have competing roles in regulating the viral life cycle, our data suggests immune evasion to be the predominant effect in the clinical setting of active viral replication. Finally, it does not appear that BKV miRNA measurements in urine provide information relevant to clinical prognosis.

Ethical approval

IRB protocol #000586, University of Pittsburgh

Funding

Department of Pathology, University of Pittsburgh.

CRediT authorship contribution statement

Yuchen Huang: Validation. **Gang Zeng:** . **Parmjeet S. Randhawa:** Funding acquisition, Conceptualization, Methodology, Supervision, Writing - original draft.

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2019.07.006.

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