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Polyomavirus BK-encoded microRNA suppresses autoregulation of viral replication

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ABSTRACT

Polyomavirus BK (BKV) infection is an important cause of renal allograft failure. Viral microRNAs are known to play a crucial role in viral replication. This study investigated the expression of BKV-encoded microRNAs (miR-B1) in patients with polyomavirus-associated nephropathy (PVAN) and their role in viral replication. Following BKV infection in renal proximal tubular cells, the 3p and 5p miR-B1 levels were significantly increased. Cells transfected with the vector containing the miR-B1 precursor (the miR-B1 vector) showed a significant increase in expression of 3p and 5p miR-B1 and decrease in luciferase activity of a reporter containing the 3p and 5p miR-B1 binding sites, compared to cells transfected with the miR-B1-mutated vector. Transfection of the miR-B1 expression vector or the 3p and 5p miR-B1 oligonucleotides inhibited expression of TAg. TAg-enhanced promoter activity and BKV replication were inhibited by miR-B1. In contrast, inhibition of miR-B1 expression by addition of miR-B1 antagomirs or silencing of Dicer upregulated the expression of TAg and VP1 proteins in BKV-infected cells. Importantly, patients with PVAN had significantly higher levels of 3p and 5p miR-B1 compared to renal transplant patients without PVAN. In conclusion, we demonstrated that (1) miR-B1 expression was upregulated during BKV infection and (2) miR-B1 suppressed TAg-mediated autoregulation of BKV replication. Use of miR-B1 can be evaluated as a potential treatment strategy against BKV infection.

1. Introduction

Reactivation of polyomavirus BK (BKV) causes devastating problems such as polyomavirus-associated nephropathy (PVAN) in kidney transplant patients and hemorrhagic cystitis in patients undergoing bone marrow transplants [1]. Although the importance and clinical outcome of PVAN is well known, there are currently no effective anti-viral agents [2–4]. It is therefore crucial to understand the mechanisms underlying BKV replication in order to develop strategies to prevent progression of PVAN.

The BKV large T antigent (TAg) binds to the origin of the viral genome and stimulates viral DNA replication by acting as a DNA helicase and regulating the expression of both early and late viral genes [5]. Recognition of TAg by cytotoxic T cells resulted in host cell lysis and virus clearance [6]. These data suggested that inhibition of TAg could result in suppression of viral growth.

MicroRNAs (miRNAs) are small non-coding RNAs that can regulate gene expression through degradation of mRNA or by translational inhibition. Like most mammalian microRNAs, viral miRNAs are processed from pre-miRNA by Dicer and incorporated into the RNA-induced silencing complex (RISC) to inhibit target gene expression [7–10]. A large number of viral miRNAs have been shown to regulate pathways involved in viral replication, anti-viral immunity or tissue tropism [11,12]. There is a growing understanding of the mechanisms by which viruses establish latency and a number of viral gene products, especially those associated with early viral replication have been shown to be down-regulated during latency via viral miRNA-mediated inhibition. For example,

Abbreviations: BKV, polyomavirus BK; KSHV, Kaposi's sarcoma-associated herpesvirus; NCCR, non-coding control region; PVAN, polyomavirus-associated nephropathy; RISC, RNA-induced silencing complex; TAg, large T antigen; VP1, viral capsid protein 1.

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Kaposi's sarcoma-associated herpesvirus (KSHV) microRNA, miRK9*, regulates lytic switch protein RTA to fine-tune lytic reactivation [13], while virus-encoded miR-H2 was shown to maintain herpes simplex virus (HSV-1) latency in neuron cells by downregulating ICP0, an early transcriptional activator for viral productive replication [14]. Monkey polyomavirus simian virus 40 encodes miRNAs that regulate early viral gene expression and reduce susceptibility to cytotoxic T cells and shares conserved functions among the primate polyomavirus' encoded miRNAs [6,15]. In this study, we aimed to investigate the role of BKV-encoded microRNA in autoregulation of viral replication.

2. Materials and methods

2.1. Cell culture

The HK-2 cells were cultured in Dulbecco's minimum essential medium/Ham's F12 (Life Technologies, Paisley, UK) supplemented with 5% fetal calf serum (FCS; Biological Industries Ltd, Cumbernauld, UK).

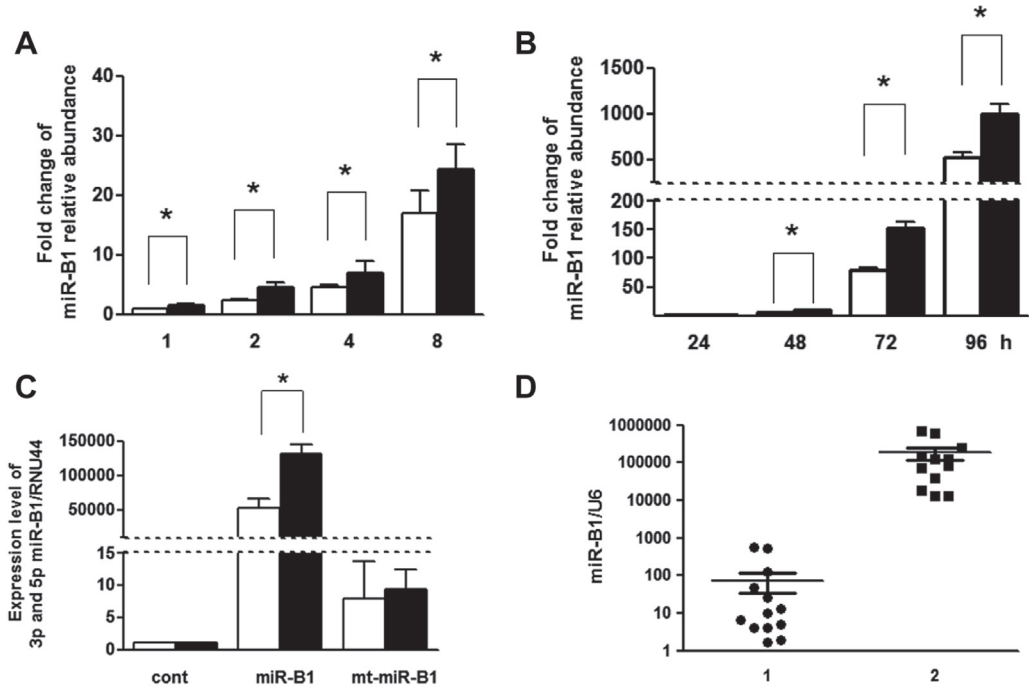
2.2. Plasmids construction

Pre-miRB1 expression vectors were generated by PCR-amplification of the sequences mapping to BKV TAg using forward primer 5-GCCGGTACCACCGAAAGCCTTTACACAAATGCAAC-3 and reverse primer 5-GCCGGATCCACCGGAAGGAAAGGCTGGATTCT-3, and inserting the fragment into pcDNA4. Expression vectors carrying specific mutations were also generated using primers designed with the appropriate mutations. A luciferase reporter plasmid containing the sequence complementary to pre-miRB1 was generated by PCR-amplifying a 200 bp fragment containing this segment along with additional flanking regions and cloning this into a pGL4 luciferase-reporter vector (Promega, Madison, WI). The BKV TAg-expressing plasmid was generated as described previously [16].

2.3. Quantitative polymerase chain reaction

Total RNA was extracted using the miRNA isolation kit (Geneaid). RNA was incubated with the reverse transcriptase master mix from the miRNA Reverse Transcription kit (ABI, USA) and the specific loop reverse transcription (RT) primers which were designed according to the microRNA database miRbase version 16: (5-TCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGACTCTGG-3; 5-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATGCTCTT-3). The reaction mix was incubated at 16 °C for 30 min and then at 42 °C for 30 min. The cDNAs from the RT reaction were subjected to real-time PCR using the following primers: miR-B1–3p: 5-CGGCGGTGCTTGATCCATGTCC-3; miR-B1–5p: 5-CGGCGGATCTGAGACTTGGGAA-3; and universal primer 5-CTGGTGTCGTGGAGTCGGCAATTC-3. MicroRNA expression levels were normalized to common internal control (small RNA RNU44).

To determine viral load, BKV and cellular DNA were extracted from cell lysate using a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). qPCR was performed as conventional protocol. The BKV DNA was normalized by analyzing samples in parallel by the qPCR for the cellular tubulin β2A DNA using the commercial prim-



miR-B1
ATCTGAGACTTGGGAAGAGCAT .....TGCTTGATCCATGTCCAGAGTC

Mutated miR-B1
A***GATCGAT***CTTGGGAAGAGCAT.....T***TGAGCTT***CCATGTCCAGAGTC

Fig. 1. miR-B1 expression is increased following BKV infection or transfection with the miR-B1 vector. (A and B) Cells were infected with BKV (1–8 × 10^7 copies/mL) for 72 h (A) or with BKV (1 × 10^7 copies/mL) for 24–96 h (B). (C) Cells were transfected with the control vector (cont), the miR-B1 vector or the miR-B1-mutated vector for 72 h. miR-B1 expression was quantified at the end of each time period. Asterisks indicate $p < 0.05$. (D) Thirteen urine samples from 6 renal transplant patients without PVAN (group 1) and twelve urine samples (group 2) from 10 renal transplant patients with PVAN were collected. Total RNA in these samples was extracted and miR-B1 expression levels were then quantified.

ers and probes (Hs00742533_s1*, probe: GTCCTCAAGCATGGTCTTTCTACTT; Applied Biosystems).

2.4. Luciferase assays

Luciferase assays were performed with the luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's recommendations. Luciferase activity (relative light units) was measured in duplicate samples using a luminometer (MLX micro titer plate luminometer, Dynex Ltd, Chantilly, VA). Data were presented as firefly luciferase activity normalized to Renilla luciferase activity.

2.5. Western blot analysis

Total cellular protein was extracted as previously described [17]. Protein samples were mixed with reducing sodium dodecyl sulfate sample (SDS) buffer and boiled for 5 min at 95 °C and then resolved on a SDS polyacrylamide gels and then electroblotted on nitrocellulose membranes. The membrane was incubated with primary antibody and subsequently horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were visualized using enhanced chemiluminescence (Amersham Bio-sciences, Amersham, UK). Mouse anti-SV40 TAg antibody was purchased from Calbiochem (La Jolla, CA). Mouse anti-VP1 antibody was obtained from Abnova (Taipei, Taiwan).

2.6. Patients

Samples were taken from renal transplant recipients in our center after the informed consent was obtained. Thirteen urine samples were obtained from six renal transplant patients with PVAN and 13 urine samples were collected from 10 renal transplant patients without PVAN.

2.7. Statistical analysis

All the data were presented as means ± standard deviation. The Student's *t*-test was applied to compare the means of miR-B1 expression. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of BKV miR-B1 expression following BKV infection

BKV pre-miRNA predicted by miRbase 16.0, are processed into two 22-nucletoide miRNAs [the 3p miRNA (3p miR-B1) and the 5p miRNA (5p miR-B1)], which are fully complementary to the BKV TAg mRNA. To ascertain if both arms of miR-B1 are expressed following BKV infection, we used stem-loop reverse transcription and qPCR to quantify expression of 3p and 5p miR-B1 in cells infected with BKV (TU strain). Both 3p and 5p miR-B1 were expressed in a dose- and time-dependent manners (Fig. 1A and B). In order to confirm that the BKV pre-miRNA is processed into miR-B1, we cloned the miRNA precursor encoding miR-B1 into a pcDNA4 vector (miR-B1 vector). In addition, the miR-B1 precursors containing the mutations corresponding to the seed sequences of miR-B1 and thus lacking complementarity to TAg mRNA, were cloned into the plasmids. Expression levels of 3p and 5p miR-B1 were quantified in cells transfected for 72 h with the miR-B1 vectors. Both 3p and 5p miR-B1 were expressed in the miR-B1 vector-transfected cells, but not in cells transfected with the miR-B1-mutated vector (Fig. 1C).

To determine if miR-B1 expression is increased in renal transplant patients with PVAN, we quantitated the expression of miR-B1 mRNA in 13 urine samples from six renal transplant patients with PVAN and in 13 urine samples from 10 renal transplant patients without PVAN. As shown in Fig. 1D, miR-B1 expression was markedly increased in patients with PVAN compared to those without PVAN.

3.2. BKV miR-B1 downregulates BKV TAg transcription and translation

In order to determine whether miR-B1 suppresses BKV replication through inhibition of BKV TAg expression, a 200 nucleotide-long region corresponding to the 3p and 5p miR-B1 binding site was cloned into a firefly luciferase reporter and cells were transfected with this luciferase reporter along with the miR-B1 expression vector or the mutated miR-B1 vector. We demonstrated a significant reduction of luciferase activity in cells co-transfected with the miR-B1 vector (Fig. 2A). In contrast, no decrease in luciferase activity was seen in cells co-transfected with the miR-B1-mutated vector and the luciferase reporter.

The vector expressing flag-tagged TAg and the miR-B1 vector were cotransfected into cells in order to assess whether miR-B1 can affect BKV TAg expression. Western blot analysis was used to determine BKV TAg expression after 72 h of transfection. There was a significant reduction in BKV TAg expression in cells cotrans-

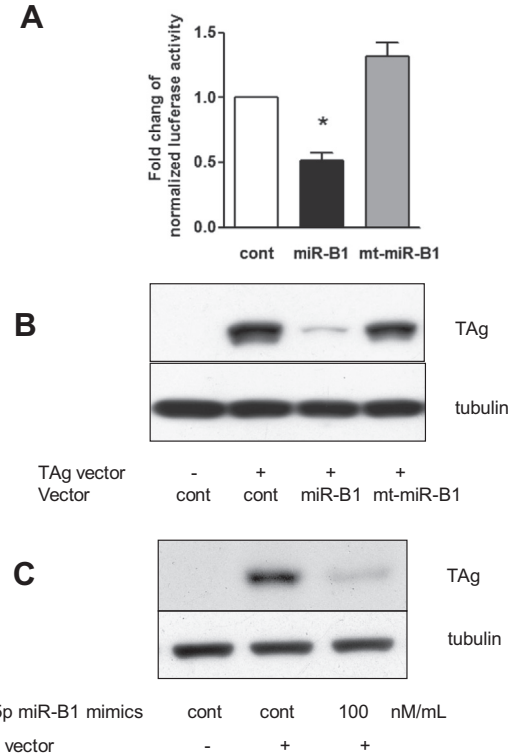


Fig. 2. BKV miR-B1 downregulates BKV TAg transcription and translation. (A) Cells were co-transfected with a firefly luciferase reporter containing the miR-B1 complementary sequence together with the control vector (cont), the miR-B1 vector or the miR-B1-mutated vector for 24 h. Firefly luciferase activity was normalized to Renilla luciferase activity (internal control). Asterisks indicate $p < 0.05$. (B) Cells were co-transfected with the TAg-expressing vector (1 μg/mL) and the miR-B1 vector (4 μg/mL) or the miR-B1-mutated vector (4 μg/mL) for 72 h. (C) A mixture of 3p miR-B1 mimic (100 nM/mL) and 5p miR-B1 mimic (100 nM/mL) was introduced into the cells. The level of TAg and tubulin (control) proteins were determined by immunoblot assay.

fected with the miR-B1 vector (Fig. 2B). In contrast, cotransfection with the miR-B1-mutated vector did not inhibit TAg expression.

To further verify the inhibitory effect of miR-B1 on BKV replication, we generated 3p and 5p miR-B1 oligonucleotides (3p and 5p miR-B1 mimics) to mimic the function of miR-B1. Cells were co-transfected with a mixture of 3p and 5p miR-B1 mimics and the BKV TAg-expressing vector. We showed that addition of the mixture of 3p and 5p miR-B1 mimics reduced TAg expression (Fig. 2C), further confirming the suppressive effect of miR-B1 on TAg expression.

3.3. miR-B1 inhibits BKV replication and TAg-mediated autoregulation

In order to evaluate if miR-B1 could suppress BKV replication, cells were transfected with the miR-B1 vector and then infected with BKV for 72 h. Cells transfected with miR-B1 had a significant reduction in BKV TAg and VP1 protein expression and viral titers, while transfection of the mutated miR-B1 vector did not significantly cause these alterations (Fig. 3A and B), suggesting that miR-B1 exerted an inhibitory effect on BKV replication. To further verify this finding, cells were transfected with the 3p miR-B1 mimic, 5p miR-B1 mimic or a mixture of 3p/5p mimics prior to infection with BKV. There was a significant inhibition of TAg and VP1 expression and viral titers in cells transfected with the 3p miR-B1 or the 5p miR-B1 mimics compared to cells transfected with the scrambled control oligonucleotides (Fig. 3C and D).

We and others previously demonstrated that binding of BKV TAg to BKV NCCR activated viral gene expression and autoregulated viral replication [16]. Overexpression of TAg increased NCCR luciferase activity, suggesting that TAg induced a positive autoregulation by activating the viral promoter. The TAg-mediated enhancement of NCCR activity was suppressed by overexpression of miR-B1 (Fig. 3E). To further verify the finding that the TAg-mediated autoregulation of viral replication is inhibited by miR-B1, cells were cotransfected with the TAg expression vector and the miR-B1 vector and then infected with BKV. We showed that over-

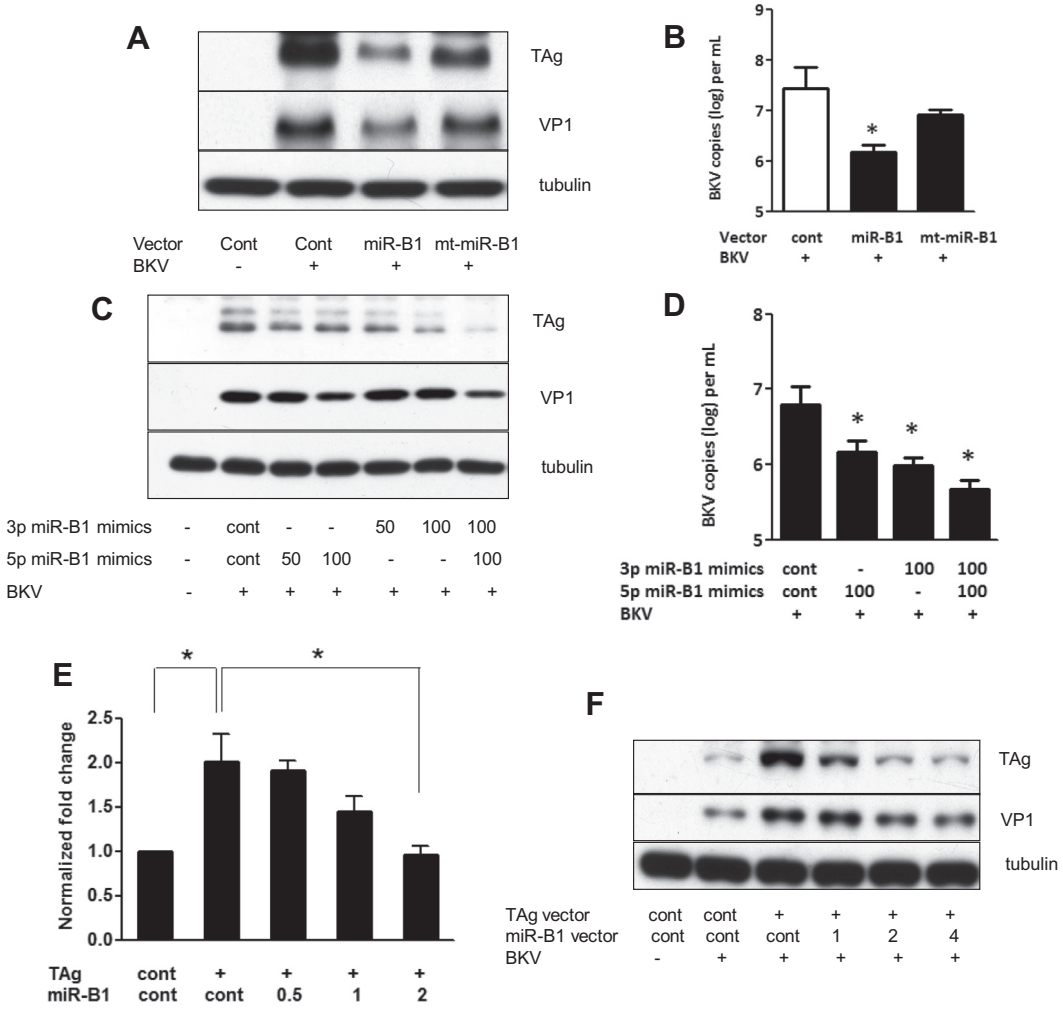


Fig. 3. miR-B1 inhibits TAg-mediated autoregulation of BKV replication. (A and B) Cells were transfected overnight with the miR-B1 vector or the miR-B1-mutated vector, and then infected with BKV (1×10^7 copies/mL) for 72 h. (C and D) The scramble control oligonucleotides (100 nM/mL), 3p miR-B1 mimic (50–100 nM/mL), 5p miR-B1 mimic (50–100 nM/mL) or the mixture of 3p and 5p miR-B1 mimics (both at 100 nM/mL) were introduced to cells prior to infection with BKV (1×10^7 copies/mL) for 72 h. The protein levels of TAg, VP1 and tubulin were assessed by Western blot analysis (A and C) and BKV titers were determined by qPCR (B and D). (E) Cells were co-transfected with the NCCR luciferase reporter (1 μg/mL), the TAg-expressing vector (1 μg/mL) and the miR-B1 vector (0.5–2 μg/mL) or the control vector (2 μg/mL) for 24 h. The firefly luciferase activity was normalized to Renilla luciferase activity. Asterisks indicate $p < 0.05$. (F) Cells were transfected with the miR-B1 vector (1–4 μg/mL) and the TAg-expressing vector (1 μg/mL) for 24 h and then infected with BKV (1×10^7 copies/mL) for 72 h. The protein levels of TAg, VP1 and tubulin were determined by immunoblot assay.

expression of TAg augmented BKV VP1 expression, while cotransfection with the TAg and miR-B1 vectors inhibited the TAg-mediated increase of VP1 expression (Fig. 3F).

3.4. Inhibition of miR-B1 by their cognate inhibitors and Dicer silencing enhances viral replication

We further investigated whether neutralization of miR-B1 by its anti-sense oligonucleotides could abrogate the suppressive effect of miR-B1 on TAg expression. The inhibitory effect of miR-B1 on TAg expression was reduced upon pre-treatment with anti-miR-B1 antagomirs (Fig. 4A). To further assess whether anti-miR-B1 antagomirs neutralized virus-produced miR-B1, thereby enhancing BKV replication, antagomirs were introduced into cells prior to infection with BKV. Results showed enhanced BKV VP1 expression and BKV titers in cells transfected with anti-miR-B1 antagomirs compared to cells transfected with control oligonucleotides (Fig. 4B and C). Dicer is essential for processing of pre-miRNA to miRNA in mammalian cells and viruses. Following knockdown of Dicer by siDicer, cells were transfected with the miR-B1 vector and the TAg-expressing vector. Knockdown of Dicer expression partially rescued the miR-B1-mediated inhibition of TAg expression (Fig. 4D). To further assess whether knockdown of Dicer reduced miR-B1 production following BKV infection, Dicer expression was knocked down by siDicer and cells were then infected with BKV. Knockdown of Dicer expression significantly reduced expression of both 3p and 5p miR-B1 (Fig. 4E) and enhanced VP1 expression (Fig. 4F).

4. Discussion

In this study, we demonstrated a significant increase in the expression levels of both 3p and 5p miR-B1 in cells infected with BKV. We also showed a significant increase in the expression levels of 3p and 5p miR-B1 in cells transfected with the vector containing the miR-B1-encoded miRNA precursor compared to cells transfected with the control vector or the miR-B1 mutated vector. Our data suggest that mature 3p and 5p miR-B1s are processed from the pre-miR-B1 precursor during BKV replication. The polyomavirus family includes microRNAs of SV40, JCV, Merkel cell polyomavirus and the murine polyomavirus [6,15,18,19]. Although the miRNAs shared the conserved function of down regulating early transcript,

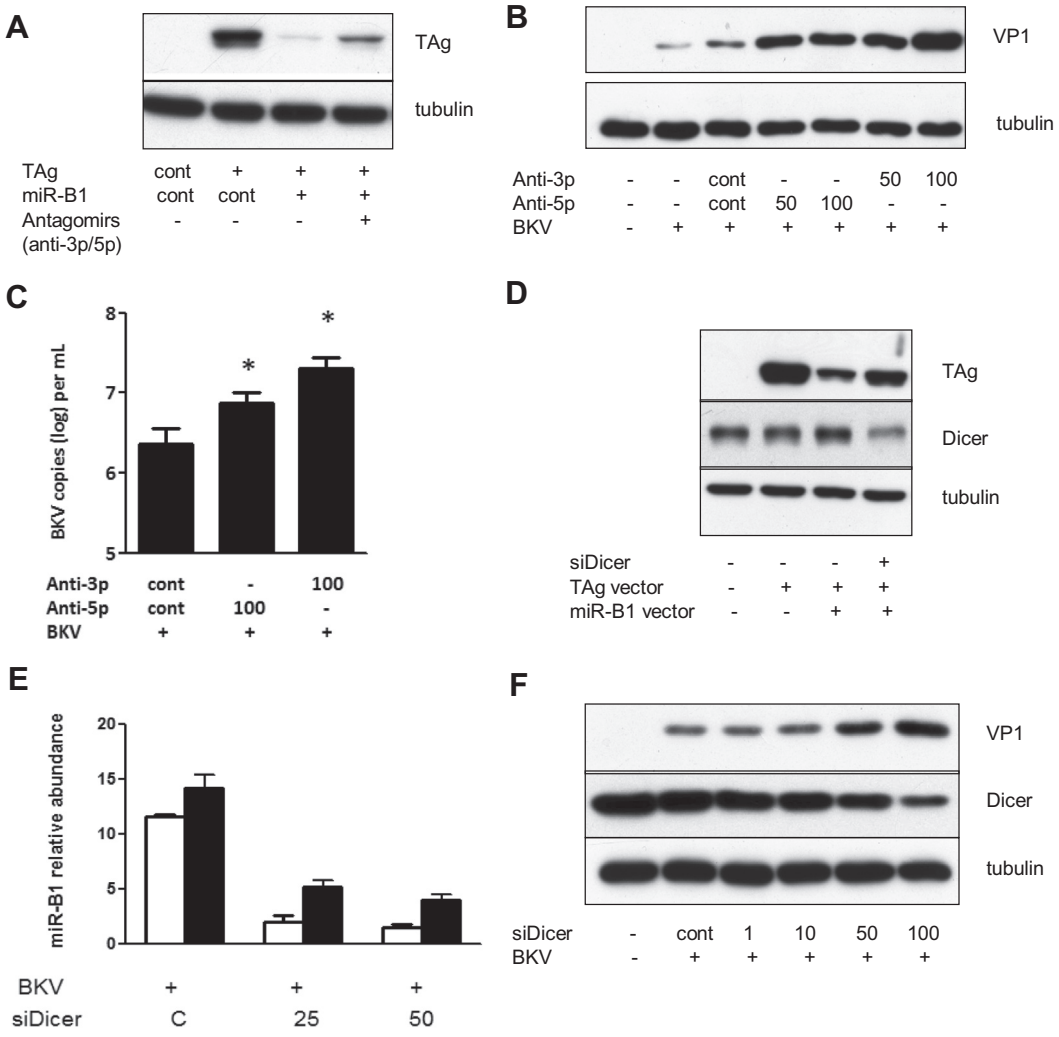


Fig. 4. Inhibition of miR-B1 expression enhanced BKV replication. (A–C) Cells were transfected with a mixture of miR-B1 antagomirs (3p miR-B1 antagomir: anti-3p 50–100 nM/mL; 5p miR-B1 antagomir: anti-5p 50–100 nM/mL) prior to co-transfection with the TAg-expressing vector and the miR-B1 vector (A) or infection with BKV (1×10^7 copies/mL). (B and C) BKV titers were determined by qPCR. (D–F) Cells were transfected with siDicer (25–50 nM/mL) overnight and then co-transfection with the TAg-expressing vector and the miR-B1 vector (D) or infected with BKV (1×10^7 copies/mL) for 72 h. (E and F) The protein levels of TAg, VP1 and Dicer were determined by immunoblot assay. (D and F) The expressions of 3p miR-B1 and 5p miR-B1 were quantified by qPCR (E).

the functionality of these individual miRNA are not all known. For example, SV 40 encodes miRNA to evade immune recognition but the murine polyomavirus encoded miRNAs are not important to experimental infection [6,19]. Seo et al. have shown that JCV and BKV generated miRNAs and JCV miRNAs inhibited early gene expression [15]. Broekema et al. have recently demonstrated that BKV encoded miRNA is implicated in the mechanism of viral persistence [20]. In our study, we reiterate the role of BKV encoded miRNA in suppression of virus replication through inhibition of TAg-mediated autoregulation.

Overexpression of miR-B1 suppressed luciferase activity of a reporter containing the 3p and 5p miR-B1 binding sites and also inhibited the expression of TAg in cells transfected with the TAg-expressing vector, confirming that miR-B1 could inhibit TAg expression. Since TAg regulates viral replication by binding to the origin of replication on NCCR and initiating DNA transcription, we speculated that inhibition of TAg expression by miR-B1 would suppress viral replication. As expected, overexpression of miR-B1 reduced TAg-augmented NCCR activity and TAg-enhanced VP1 expression, suggesting an inhibitory effect of miR-B1 on the TAg-mediated autoregulation of BKV replication. Ovexpression of miR-B1 prior to BKV infection also reduced viral VP1 expression. Our results indicated that BKV replication was down-regulated by its own microRNAs through inhibition of TAg expression.

BKV is known to establish a lifelong persistent infection in the epithelial cells of the renourinary tract and remain latent in immunocompetent subjects. BKV reactivation in immunocompromised patients was shown to result in a lytic infection, leading to further infection of neighboring cells. The switch between latency and lytic infection is essential for pathogenic viruses to evade immune clearance and spread from cell to cell [5,21]. Since latency is fundamental for viruses to survive in the host, viruses adapt in different ways, including restriction of viral gene expression and modification of host cellular processes to avoid immune clearance [22]. Recently, virus-encoded miRNAs have been reported to regulate both viral and cellular gene expression in favor of the switch of the life cycle [23]. Bellare et al. have demonstrated that the Kaposi's sarcoma-associated herpesvirus-encoding miRNAs affects the latent-lytic switch via regulation of the replication and transcription activator (RTA) [13]. In our study, miR-B1 suppressed TAg expression and TAg-mediated autoregulation of BKV replication. Knockdown of miR-B1 by the antagomirs enhanced viral replication. We speculate that viral replication during active infection is driven by the strong transactivation of TAg by host transcription factors, which overrides the inhibitory effect of miR-B1. In contrast, during latent infection, TAg-mediated autoregulation of viral replication is partly suppressed by miR-B1, leading to attenuation of rapid viral replication. In line with the ideas, a recent study demonstrated that the relative levels of miRNA and TAg determined the potential of BKV replication [20]. It is important to further elucidate the mechanisms and regulators involved in the switch between latency and activation.

We showed that suppression of miR-B1 expression by silencing Dicer resulted in increased expression of VP1, underlining the importance of miR-B1 expression in inhibition of BKV replication. Our data also suggests a role for Dicer in generation of miR-B1 and in regulation of viral replication. Dicer has previously been shown to play an essential role in processing of viral microRNAs [24–26]. Interestingly, silencing of Dicer has been shown to enhance HIV-1 replication [27]. Klase et al. demonstrated that Dicer cleaves the HIV-1 TAR elements to generate TAR-derived miRNA, which inhibits HIV-1 gene expression [28]. These studies suggest that Dicer is essential for generation of viral microRNA and for regulation of viral replication. Although our data suggest a role for Dicer-mediated miR-B1 processing in the regulation of BKV replication, it is important to further elucidate whether host microRNAs processed by Dicer also affect BKV replication.

microRNAs have recently been used as targets of therapeutic agents and as diagnostic tools in patients with virus-associated diseases. A microRNA-122 antagomir consisting of a locked nucleic acid–modified phosphorothioate oligonucleotide (SPC3649), was successfully used to treat chronic HCV infection in chimpanzees, without evidence of viral resistance [29]. Lentiviral delivery of microRNAs has also been found to be a potential therapeutic strategy for NPC [30]. Our present study demonstrated that miR-B1 suppressed VP1 expression and that administration of a mixture of 3p and 5p miR-B1 mimics reduced BKV replication. There is currently no effective therapeutic strategy to prevent progression of PVAN, and our data showing that inhibition of the TAg expression by miR-B1 or its mimics to block BKV autoregulation might represent a potentially therapeutic approach in the therapy of BKV infections.

In this study, miR-B1 was detected in the urine of renal transplant patients with PVAN but not in renal transplant patients without PVAN, suggesting that urinary miR-B1 expression may be a potential biomarker to monitor the development of PVAN. Our data may reflect active replication of BKV accompanied by an increase of miR-B1 expression, which may suppress and limit over-replication of BKV. However, we cannot exclude the possibility that miR-B1 may regulate host factors which play a role in the development of PVAN and therefore contribute to the progression of PVAN. Plasma EBV encoded microRNAs have been used in nasopharygeal carcinoma (NPC) screening especially when EBV DNA is not detectable [20]. Whether urinary miR-B1 expression level can be used as a biomarker of PVAN requires further investigation.

In conclusion, this study demonstrated that upregulation of miR-B1 expression during BKV infection is accompanied by suppression of TAg-mediated autoregulation of BKV replication. Addition of miR-B1 mimics reduced BKV TAg and VP1 expression, whereas inhibition of miR-B1 expression by the cognate antagomirs or by silencing of Dicer expression enhanced BKV replication. Whether miR-B1 or its mimics can be used as a potential anti-BKV treatment remains an intriguing question which needs to be addressed.

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