Antiviral Research 103 (2014) 25–31

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Inhibition of BK virus replication in human kidney cells by BK virus large tumor antigen-specific shRNA delivered by JC virus-like particles

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ARTICLE INFO

Article history:
Received 27 October 2013
Revised 25 December 2013
Accepted 27 December 2013
Available online 7 January 2014

Keywords:
BK polyomavirus
JCPyV VLP
Short hairpin RNA
BKPyV replication
Gene therapy

ABSTRACT

Polyomavirus-associated nephropathy (PVAN) due to lytic infection by the BK polyomavirus (BKPyV) remains an important cause of allograft dysfunction and graft loss in renal transplant recipients. PVAN is commonly treated by reducing the dosage of immunosuppressive drugs and adding adjuvant antiviral agents, but the outcomes have been less than satisfactory. The BKPyV early protein large tumor antigen (LT) is indispensable for viral genome replication and viral late protein expression. Therefore, suppressing LT expression may be a way to inhibit BKPyV replication without harming the host human kidney cells. Previous studies have shown that JC polyomavirus (JCPyV) virus-like particles (VLPs), which have tropism for the human kidney, can package and transfer exogenous genes into human kidney cells for expression. In this study, we constructed an expression plasmid for a BKPyV LT-specific shRNA (shLT) and used JCPyV VLPs as a delivery vehicle to transduce the shLT plasmid into BKPyV-infected human kidney cells. The expression of BKPyV early (LT) and late (VP1) proteins was examined after transduction by immunofluorescence microscopy and Western blotting. We found that transduction with the shLT plasmid decreased the proportions of BKPyV LT- and VP1-expressing cells by 73% and 82%, respectively, relative to control. The viral genomes were also decreased by 56%. These results point to the promising possibility of developing shLT-transducing JCPyV VLPs as a specific anti-BKPyV approach for PVAN treatment.

1. Introduction

The human polyomavirus BK virus (BKPyV) is found in more than 90% of immunocompetent individuals, in which the virus is latent in kidney and urinary tract cells and causes no disease symptoms (Chesters et al., 1983; Heritage et al., 1981; Knowles, 2006; Knowles et al., 2003). However, in immunosuppressed individuals, BKPyV can reactivate, leading to diseases such as polyomavirus-associated nephropathy (PVAN) in kidney transplant patients (Hirsch et al., 2002; Purighalla et al., 1995) or hemorrhagic cystitis in bone marrow transplant patients (Dropulic and Jones, 2008; Hashida et al., 1976). PVAN is an important infectious complication affecting 1–10% of renal transplant patients (Hirsch et al., 2005). The majority of PVAN cases occur within one year post transplantation and cause allograft failure in more than half of the cases (Hirsch et al., 2005; Ramos et al., 2002; Wen et al., 2007). More than 95% of PVAN cases are caused by BKPyV (Chen et al., 2001; Namba et al., 2005; Randhawa et al., 2002a,b); fewer than 5% of the PVAN cases are related to another polyomavirus, the JC polyomavirus (JCPyV) (Baksh et al., 2001; Hurault de Ligny et al., 2000; Kazory et al., 2003; Wen et al., 2004). There is currently no effective method to treat BKPyV-related PVAN, which is commonly managed by reductions in the dosage of immunosuppressive drugs, sometimes in combination with adjuvant antiviral agents such as cidofovir, leflunomide, and fluoroquinolones (Kuypers, 2012), but the results have been less than satisfactory. Furthermore, although such approaches, by enhancing the patients' own immunity against infections, can perhaps limit the rate of BKPyV replication, they can also raise the risks of acute rejection and persistent alloimmune injury. Also, nonspecific adjuvant antiviral therapy may cause liver and kidney toxicity in renal transplant patients, a concern that speaks to the need to develop specific antiviral therapies for PVAN.

BKPyV, a member of the family *Polyomaviridae* (Hirsch, 2010), contains a double-stranded DNA genome and was first isolated in 1971 (Gardner et al., 1971). During the early phase of BKPyV

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http://dx.doi.org/10.1016/j.antiviral.2013.12.013

infection, the viral regulatory protein large tumor antigen (LT) is made in the host cell. LT expression is absolutely required for viral DNA replication and for the expression of viral structural proteins in the late phase of infection, pointing to the essential role of LT in the viral life cycle (Cassill et al., 1989; Harris et al., 1998). Consequently, inhibition of BKPyV LT expression will effectively suppress BKPyV replication. The RNA interference (RNAi) mechanism, discovered in 1998 (Fire et al., 1998), presents a strategy for the highly specific inhibition of target proteins at the gene expression level. RNAi in the form of a plasmid that expresses a specific short hairpin RNA (shRNA) can provide long-lasting inhibition (Brummelkamp et al., 2002). Thus, it should be possible to inhibit BKPyV LT expression by using the shRNA approach. However, the usefulness of this approach depends on the availability of means to successfully and efficiently deliver RNAi into cells (Pecot et al., 2011). A previous study from our laboratory has shown that the JCPyV major structural protein, VP1, can be expressed in *Escherichia coli* and self-assemble into virus-like particles (VLPs) that maintain the human kidney tropism of JC virions (Ou et al., 1999). As VP1 assembles into capsids, it can also package plasmids present in the *E. coli* expression system similarly to the packaging of viral genomes, allowing the resulting VLPs to deliver exogenous genes into the natural host of the virus for expression (Wang et al., 2004). The effectiveness of gene delivery by these JCPyV VLPs has been demonstrated in an animal model of colon cancer, in which the VLPs were used to package and transfer a suicide gene (Chen et al., 2010), suggesting that the JCPyV VLP can potentially serve as a gene delivery vector for gene therapy. Therefore, in this study, we wished to test the human kidney-tropic JCPyV VLP as a specific, noncytotoxic vector for delivering a BKPyV LT-specific shRNA expression plasmid for the inhibition of BKPyV replication, in order to evaluate the VLP's potential as a therapeutic agent for PVAN.

2. Materials and methods

2.1. Construction of plasmid DNA for expressing BKPyV LT-specific shRNA (shLT)

For the expression of BKPyV LT-specific shRNA, RNAi-Ready pSIREN-Shuttle (Clontech) was used as the vector plasmid, and the sequence of the LT-specific siRNA (Radhakrishnan et al., 2004) was redesigned as a shRNA sequence that can base-pair with the target region of the BKPyV LT sequence (strain UT, GenBank accession no. DQ305492, nt 4430–4448, 5′-GTC TTT AGG GTC TTC TAC C-3′). The shRNA coding sequence was annealed from two oligodeoxynucleotides, 5′-GAT CCG GTA GAA GAC CCT AAA GAC TTC AAG AGA GTC TTT AGG GTC TTC TAC CTT TTT TAA GCT TG-3′ (underlined: target sense sequence) and 5′-AAT TCA AGC TTA AAA AAG GTA GAA GAC CCT AAA GAC TCT CTT GAA GTC TTT AGG GTC TTC TAC CG-3′, and ligated into the vector plasmid via BamHI- and EcoRI-cohesive ends by using T4 DNA ligase (Promega) to generate the plasmid for expressing LT-specific shRNA, pSIREN-shLT. To generate the plasmid for expressing the scrambled counterpart of shLT, or pSIREN-scramble-shLT, the oligodeoxynucleotides 5′-GAT CCG AAC GGT CAC ACG ATA GAA TTC AAG AGA TTC TAT CGT GTG ACC GTT CTT TTT TAA GCT TG-3′ and 5′-AAT TCA AGC TTA AAA AAG AAC GGT CAC ACG ATA GAA TCT CTT GAA TTC TAT CGT GTG ACC GTT CG-3′ were annealed and ligated into the vector plasmid.

2.2. Propagation and purification of BKPyV

The UT strain of BKPyV, a gift from Dr. W. Atwood (Brown University, Rhode Island, USA), was propagated in the HK-2 human kidney cell line (ATCC, cat. no. CRL-2190). The process of BKPyV purification has been described previously (Fang et al., 2010). Briefly, BKPyV-infected HK-2 cells were suspended in phosphate buffered saline (PBS) and lysed through three cycles of freezing and thawing at −80 and 37 °C, respectively. Then BKPyV particles were released from cellular membranes by treatment with type V neuraminidase (Sigma) and purified by sedimentation through a 20% sucrose cushion.

2.3. Transfection of pSIREN-shLT into BKPyV-infected HK-2 cells

Three days after HK-2 cells were infected with 10×2^8 HA units BKPyV in 35 mm culture dishes, the cells were transfected with 5 μg pSIREN-shLT using Lipofectamine 2000 reagent (Invitrogen). BKPyV LT expression was analyzed on day 3 posttransfection by immunofluorescence microscopy.

2.4. Immunofluorescence microscopy

Cells on a coverglass were fixed with cold methanol and acetone at a 2:1 volume ratio. The coverglass was blocked with normal horse serum (Gibco), and the cells' morphology was visualized by staining with Evans Blue solution. The cells were then incubated with mouse monoclonal antibody against SV40 LT (Calbiochem), which cross-reacts with BKPyV LT, or with rabbit polyclonal antibody against BKPyV VP1, for detecting viral early or late protein, respectively. The primary antibody incubation was followed by incubation with FITC-conjugated goat anti-mouse IgG (Sigma) or with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes), respectively. Two thousand cells per sample type were counted under a confocal microscope (LSM 510; Carl Zeiss) for determining the percentage of positive cells.

2.5. Packaging of exogenous plasmid DNA into JCPyV VLPs in E. coli

To *in vivo* package pSIREN-shLT into JCPyV VLPs, the JCPyV VP1 expression plasmid ΔpFlag-JCPyV-VP1 and pSIREN-shLT were co-transformed into JM109 *E. coli*. Bacterial cells with the simultaneous presence of both plasmids were selected for by plating on an LB plate containing both kanamycin (to which pSIREN-shLT confers resistance) and ampicillin (to which ΔpFlag-JCPyV-VP1 confers resistance). The purification of JCPyV VLPs has been described previously (Fang et al., 2012). In brief, IPTG-induced bacteria were lysed, and a supernatant containing soluble proteins was collected by centrifugation. Then JCPyV VLPs containing packaged plasmid DNA, either pSIREN-shLT (shLT-VLPs) or pSIREN-scramble-shLT (scramLT-VLPs) were purified by 10–30% sucrose gradients and concentrated by filtration in Centricon units (Millipore).

2.6. Pseudoinfection of shLT-VLPs into BKPyV-infected HK-2 cells

Three days after HK-2 cells were infected with 10×2^8 HA units BKPyV, the cells were pseudoinfected with the shLT-VLPs as follows. The cells were washed with cold PBS, and then 20 μg of shLT-VLPs was added to the 35 mm dish of cells and allowed to incubate for 1 h at 4 °C. Afterwards, the cells were washed with cold PBS to remove any free VLPs, complete medium was added to the culture dish, and the cells were incubated at 37 °C and 5% CO_2 for 4 days.

2.7. Western blotting

Samples of cell lysates were resolved by 12.5% SDS–PAGE, and transferred onto a PVDF membrane (Pall). The membrane was blocked with 5% nonfat milk overnight and incubated with anti-SV40 LT mouse monoclonal antibody or anti-BKPyV VP1 rabbit polyclonal antibody, followed by horseradish peroxidase-labeled secondary antibody against mouse or rabbit IgG (Santa Cruz

Biotechnology), for the detection of viral early or late protein, respectively. Signal was developed by using Western Lightning Chemiluminescence Reagent Plus Kit (Perkin Elmer Life Sciences).

2.8. Quantitative PCR

To determine the viral road in cells after treatment of shLT-VLPs, the total DNA was extracted from HK-2 cells after 4-day inhibition using a QIAamp DNA Mini Kit (Qiagen). Real-time PCR was performed with primers, 5′-CAC TTT TGG GGG ACC TAG T-3′ (PoL1s) and 5′-CTC TAC AGT AGC AAG GGA TGC-3′ (PoL2as), which franking the BKPyV VP2 coding region(Whiley et al., 2001). Beta-actin, a housekeeping gene, was amplified for normalization. Primers for beta-actin amplification were 5′-TGC GTG ACA TTA AGG AGA AG-3′ (sense) and 5′-GCT CGT AGC TCT TCT CCA-3′ (antisense). The qPCR mixture contained 20 ng DNA, 10 μM of each primer and SYBR Green Real time PCR Master Mix (Toyobo). The fluorescent intensity was detected by Applied Biosystems StepOne Real-Time PCR Systems.

3. Results

3.1. Construction and functional confirmation of the BKPyV LT-specific shRNA expression plasmid (shLT)

In order to inhibit the replication of BKPyV, we needed to select a region in the gene sequence of BKPyV LT as the target for RNAi-mediated inhibition. In their 2004 publication, Khalili's group describe an siRNA fragment that inhibited the expression of JCPyV LT (Radhakrishnan et al., 2004). Because BKPyV LT has 90% homology with JCPyV LT, we chose a conserved region in the BKPyV LT gene, nt 4430–4448 for the UT strain, that corresponds to the JCPyV LT gene region targeted by their siRNA. To target this 19 nt region of the BKPyV LT transcript, our shRNA design consists of this 19 nt sequence connected to a short spacer, followed by the reverse complement of the same 19 nt sequence, so that the entire RNA expressed will fold back onto itself to form an shRNA structure. We cloned this BKPyV LT-specific shRNA coding sequence into the RNAi-Ready pSIREN-Shuttle vector and confirmed the sequence of the resulting plasmid (pSIREN-shLT) before proceeding with its functional analysis.

First, we asked whether pSIREN-shLT can inhibit the expression of BKPyV LT. Three days after HK-2 human kidney cells were infected with BKPyV, we transfected the cells with pSIREN-shLT to express the BKPyV LT-targeting shRNA inside these cells. The cells were harvested at 3 days posttransfection (Fig. 1A) and processed for immunofluorescence analysis for the expression of the viral early protein LT. As shown in Fig. 1B, the abundance of LT-positive cells among the BKPyV-infected HK-2 cells was markedly decreased by treatment with pSIREN-shLT. After quantification, the proportion of LT-positive cells was found to be 53% lower among BKPyV-infected cells treated with pSIREN-shLT than among those treated with the negative control (Fig. 1C), indicating that our BKPyV LT-specific shRNA expression plasmid does have an inhibitory effect on the production of the BKPyV early protein.

3.2. Preparation of JCPyV VLPs packaging pSIREN-shLT

Because bacterially produced JCPyV VLPs have tropism for the human kidney, we wished to use them as a delivery vector for pSIREN-shLT. In order to ensure that the JCPyV VLPs packaged pSIREN-shLT as they assembled in *E. coli*, both ΔpFlag-JCPyV-VP1, which expresses the JCPyV major structural protein, and pSIREN-shLT needed to be in the *E. coli* system simultaneously. We therefore transformed both plasmids together into *E. coli* by heat shock. Then IPTG was added to induce the *tac* operon driving VP1 expression, allowing the VP1 produced to self-assemble and package the pSIREN-shLT plasmid into shLT-VLPs. After the VLPs had been purified, we performed a hemagglutination assay to confirm the formation of JCPyV virus-like capsids, and extracted and sequenced the DNA from the capsids to confirm the presence of pSIREN-shLT in the VLPs (data not shown).

3.3. Inhibition of BKPyV replication by shLT-VLP

In order to determine if shLT-VLPs can inhibit BKPyV replication in kidney cells, HK-2 human kidney cells were used for the investigation. After HK-2 cells were infected with BKPyV (Fig. 2A), we pseudoinfected the cells with the human kidney-tropic shLT-VLPs. As detected by immunofluorescence staining after treatment with shLT-VLPs, the proportion of LT-positive cells among the BKPyV-infected cells was significantly reduced by 73% relative to that observed after treatment with controls (Fig. 2B and D). This result shows that shLT-VLPs can indeed enter human kidney cells and deliver pSIREN-shLT into the cells, thus allowing the BKPyV LT-specific shRNA to be expressed to inhibit the production of the BKPyV early protein. Infection by BKPyV causes the host cell' resources to be diverted to express the viral proteins needed for viral replication. Since the expression of viral structural proteins in the late phase, essential for the packaging of progeny viral particles, relies on not only host resources but also the BKPyV early regulatory protein LT, inhibiting LT expression can be expected to inhibit the expression of the viral structural proteins and hence the production of the viral progeny. Consistently, immunofluorescence analysis of BKPyV-infected cells showed that treatment with shLT-VLPs reduced the proportion of VP1-positive cells by 82% relative to treatment with controls (Fig. 2C and E). Furthermore, the inhibitory effect of shLT-VLPs on the expression of early and late proteins could be detected by Western blotting as substantial reductions in LT and VP1 protein levels (Fig. 3). To further demonstrate the reduction of viral load, quantitative PCR was performed. Results show that the viral genomes were decreased by 56% after the inhibition (Fig. 4). The above results demonstrate that transduction with shLT-VLPs can effectively inhibit BKPyV replication in BKPyV-infected kidney cells.

4. Discussion

In this study, we designed an shRNA construct, pSIREN-shLT, that can inhibit BKPyV early protein production, confirmed its functional activity, and placed it into an *E. coli* expression system together with an expression plasmid for the JCPyV major capsid protein VP1 (△pFlag-JCPyV-VP1). Following induction of VP1 production, VP1 self-assembly and packaging of pSIREN-shLT occurred simultaneously, generating shLT-VLPs. These shLT-VLPs could enter BKPyV-infected kidney cells and express the LT-specific shRNA, thereby inhibiting the production of BKPyV progeny and preventing the lysis of the kidney cells due to extensive viral replication. This system may offer a new approach to the treatment of PVAN.

The number of kidney disease patients receiving renal transplants is increasing annually worldwide, and the success of these transplants depends on the use of immunosuppressive regimens to prevent the acute immune reactions caused by human leukocyte antigen (HLA) mismatches (Hariharan et al., 2000). Although immunosuppression effectively lowers the rate of allograft rejection, it also predisposes the patients to infections, making infections the second leading cause of death among renal transplant recipients (Sayegh and Carpenter, 2004). In the past 20 years, the emergence of PVAN has posed a serious threat to kidney transplantation and coincided with the beginning of the widespread use of

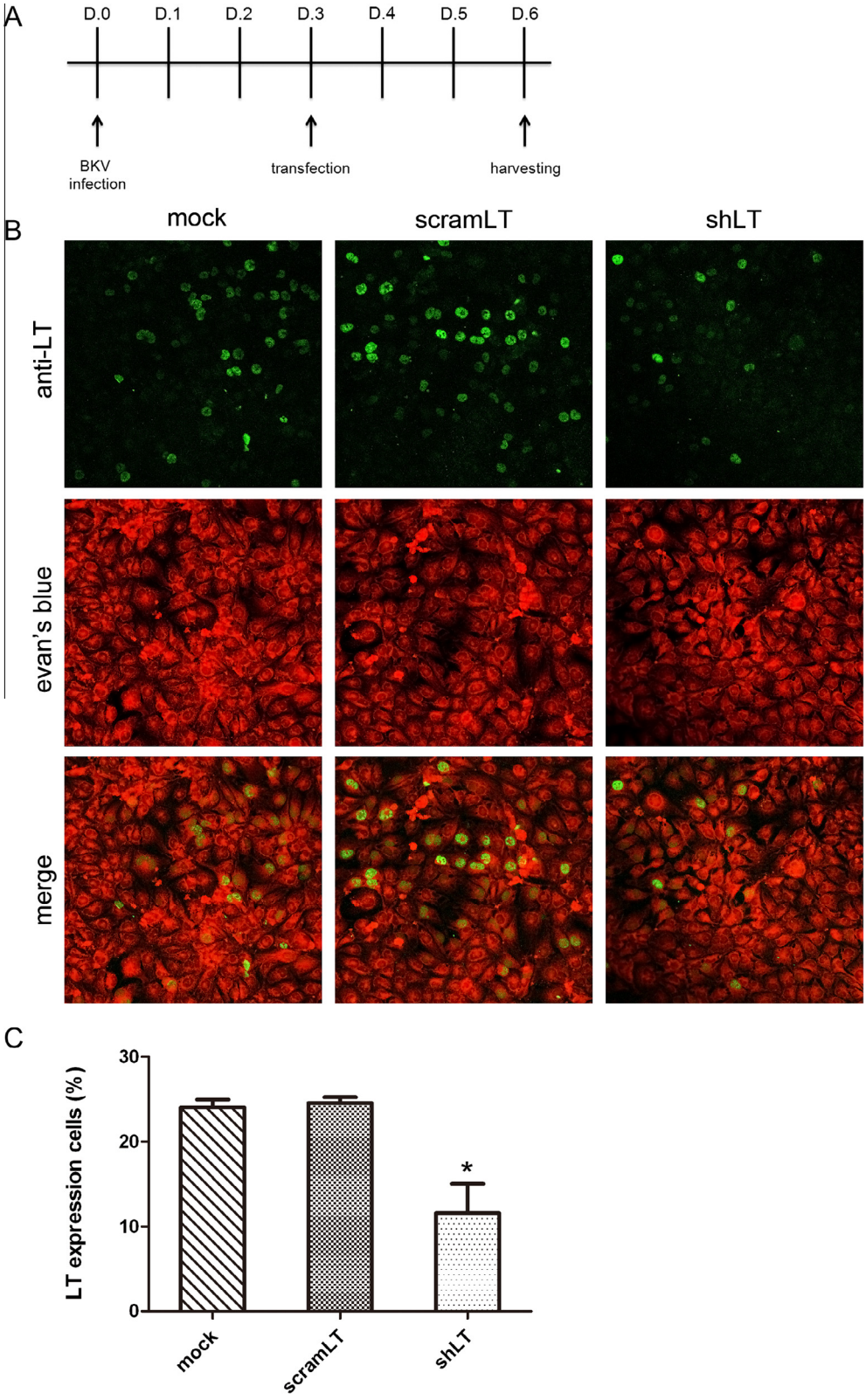

Fig. 1. Confirmation of activity of LT-specific shRNA in BKPyV-infected HK-2 cells. The expression of LT in BKPyV-infected HK-2 cells was analyzed by immunofluorescence microscopy after transfection with pSIREN-shLT (*shLT*), pSIREN-scramble-shLT (*scramLT*), and transfection reagent only (*mock*). (A) Timeline of infection and transfection. (B) Representative fields showing LT-positive cells detected by LT immunofluorescence. (C) Quantification of LT-positive cells from three independent experiments. Means and standard deviations are shown. $^{*}P < 0.0005$ by 1-way ANOVA.

many newer immunosuppressants, such as tacrolimus, mycophenolate mofetil, and sirolimus (Hirsch et al., 2005; Ramos et al., 2009). BKPyV persists in a latent state in healthy humans and increases its replication capacity in immunocompromised patients (Funk et al., 2008). Sustained immunosuppressant therapy can cause BKPyV to reactivate in renal transplant patients, leading to

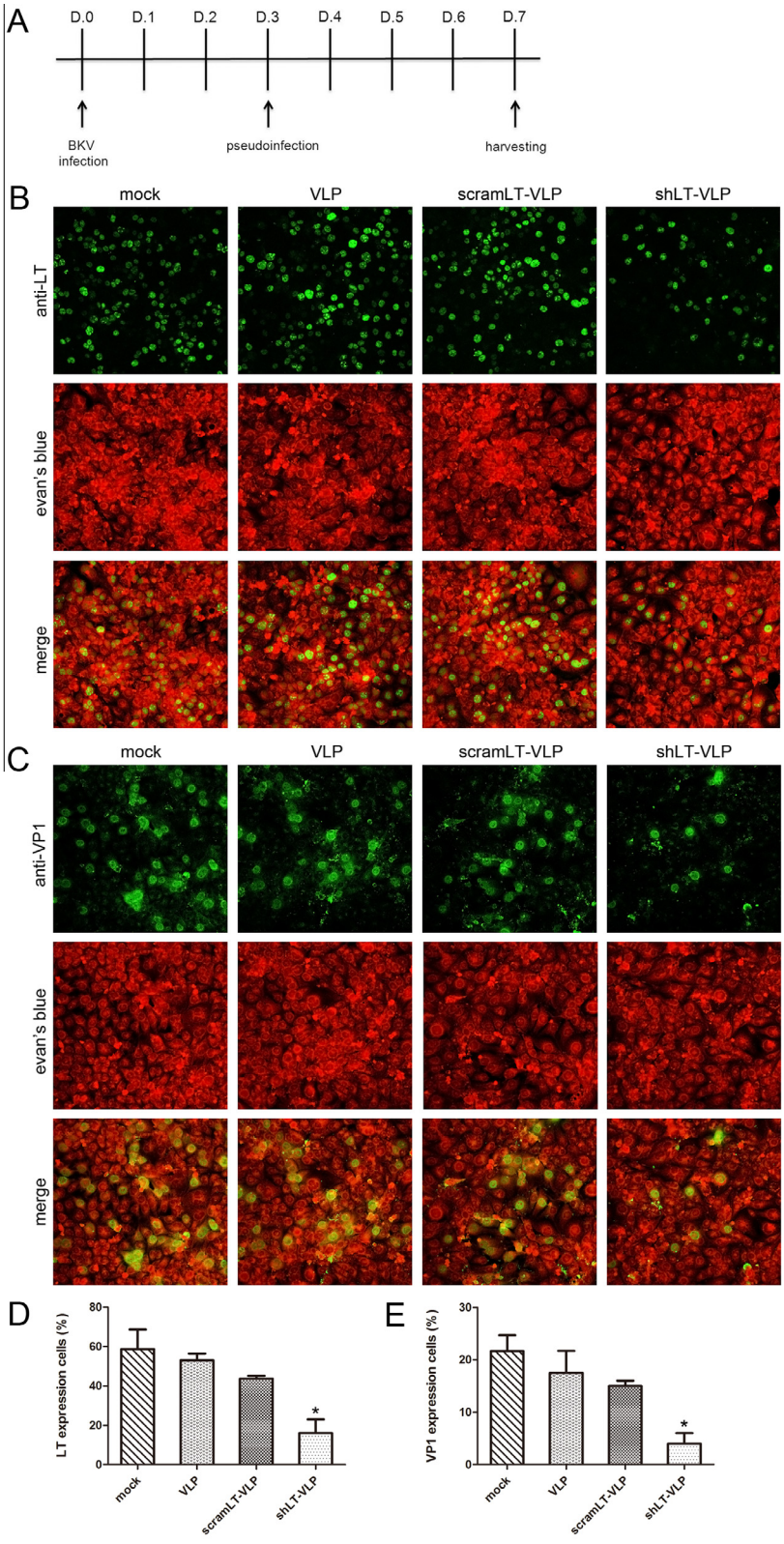

Fig. 2. Effect of JCPyV VLP-delivered LT-specific shRNA plasmid on LT and VP1 expression in BKPyV-infected HK-2 cells. The expression of LT and VP1 in BKPyV-infected HK-2 cells was analyzed by immunofluorescence microscopy after pseudoinfection with shLT-VLPs (*shLT-VLP*), scramLT-VLPs (*scramLT-VLP*), empty VLPs (*VLP*), or PBS only (*mock*). (A) Timeline of infection and pseudoinfection. (B and C) Representative fields showing cells detected by LT (B) and VP1 (C) immunofluorescence. (D and E) Quantification of the means of BKV LT- (D) and VP1- (E) positive cells. Error bars represent standard deviations. $^{*}P < 0.0005$ by 1-way ANOVA. Experiments were done in triplicate.

the development of PVAN in 1–10% of the patients and causing graft loss in approximately 50% of this subgroup. For this reason, upon detection of positive BKPyV viruria and viremia in a transplant patient, the first step to consider is to reduce the level of immunosuppression, including changing cyclosporin A to tacrolimus, reducing the dosage of calcineurin inhibitors such as mycophenolate

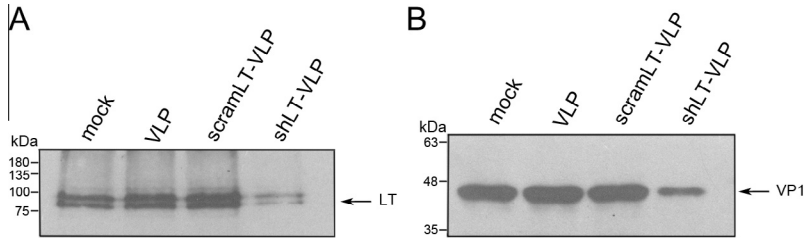

Fig. 3. Effect of JCPyV VLP-delivered LT-specific shRNA plasmid on LT and VP1 protein levels in BKPyV-infected HK-2 cells. Western blots were performed for visualizing LT and VP1 protein levels in BKPyV-infected HK-2 cells after pseudoinfection with shLT-VLPs (*shLT-VLP*), scramLT-VLPs (*scramLT-VLP*), empty VLPs (*VLP*), or PBS only (*mock*). Viral proteins were detected by using anti-LT (A) and anti-VP1 (B) antibodies.

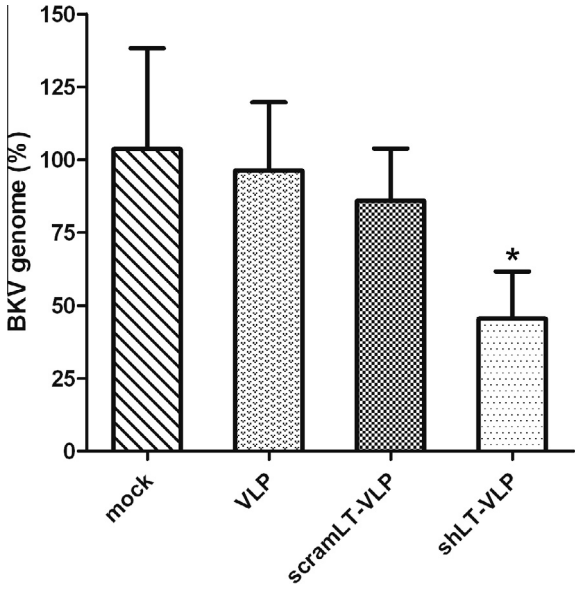

Fig. 4. Determination of BKPyV viral load by quantitative PCR. Quantitative PCR was performed to determine viral load in BKPyV-infected HK-2 cells after pseudoinfection with shLT-VLPs (*shLT-VLP*), scramLT-VLPs (*scramLT-VLP*), empty VLPs (*VLP*), or PBS only (*mock*). Means and standard deviations are shown. $^{*}P < 0.001$ by 1-way ANOVA. Experiments were done in triplicate.

mofetil or azathioprine, and halting triple drug therapies (Hirsch et al., 2005; Kuypers, 2012). This treatment strategy may also lead to acute rejection and persistent alloimmune injury in some patients due to individual physiological differences, presenting a difficult dilemma in applying this treatment approach.

When disease has progressed to PVAN, treatment typically involves reductions in immunosuppressant drugs in combination with adjuvant antiviral agents. Although these agents have not been approved by the U.S. Food and Drug Administration for PVAN treatment, they have been in clinical use for many years. Cidofovir, a monophosphate nucleotide analog, has been widely used to treat infections by a number of DNA viruses, and has been shown in an *in vitro* experiment to inhibit the replication of polyomaviruses, also DNA viruses (Andrei et al., 1997). However, the mechanism of this inhibition is unclear because polyomaviruses do not express cidofovir's target molecule, or viral DNA polymerase. Also, cidofovir is known to be nephrotoxic, making its use in renal transplant patients a concern. Leflunomide is an immunosuppressive drug whose active metabolite, A771726, has protein kinase inhibition and pyrimidine depletion activities and is therefore thought to inhibit polyomavirus replication (Williams et al., 2005). However, leflunomide is hepatotoxic, necessitating frequent patient monitoring during its use. Fluoroquinolones, a family of antibiotics, target bacterial topoisomerases II and IV and are also believed to inhibit the helicate activity of polyomavirus LT and thus reduce viral replication (Sharma et al., 2011). All the antiviral therapies mentioned above have little specificity for polyomaviruses and do not produce sufficient therapeutic effect. Nevertheless, these therapies have been in clinical use on an empirical basis for many years because specific treatments for PVAN have yet to be developed.

The discovery of RNAi, a mechanism by which target genes can be specifically inhibited, is a revolutionary development that can generate new strategies of medical interventions (Fire et al., 1998). An RNAi strategy has been used to inhibit the expression of polyomavirus proteins in order to block progeny virus production (Orba et al., 2004; Radhakrishnan et al., 2004). Compared to small interfering RNA (siRNA), which has a relatively short half-life because of serum endonucleases and rapid renal clearance (Pecot et al., 2011), shRNA expressed from plasmid DNA is a more stable and durable form of RNAi (Brummelkamp et al., 2002) and thus permits broader and more efficient application of RNAi. The JCPyV VLP, with tropism for the human kidney, would make an excellent gene delivery vector for shRNA plasmids. Recently, the method of *in vivo* packaging has been developed (Chen et al., 2010), resulting in increased packaging efficiency and a higher DNA packaging capacity (Fang et al., 2012). When JCPyV VLPs carrying a suicide gene were injected into mice in an animal model of human colon cancer, tumor size was dramatically reduced (Chen et al., 2010), demonstrating the effectiveness of the JCPyV VLP as a gene transfer vector (Chang et al., 2011). A high transduction efficiency, a human kidney tropism, and the ability to encapsidate whole plasmids make the JCPyV VLP an excellent vehicle for the targeted delivery of the BKPyV LT-specific shRNA expression plasmid into human kidney cells.

In the polyomavirus-infected host cell, the virally encoded early protein LT is a multifunctional regulatory protein whose origin-binding domain binds to the replication origin of the viral DNA and whose helicase activity initiates viral DNA replication and promotes the expression of viral late genes. There are also many recent cases of renal transplant patients for whom immunosuppression-induced polyomavirus reactivation was a possible cause of their urothelial carcinoma (Alexiev et al., 2013; Bialasiewicz et al., 2013; McDaid et al., 2013; Pino et al., 2013; van Aalderen et al., 2013). In short, while polyomaviruses latent in healthy humans cause no disease, they become a risk factor for serious disease in immunocompromised patients such as renal transplant recipients. The multifunctionality of viral LT makes it an excellent target for the development of specific antiviral drugs for treating polyomavirus-mediated diseases, and our shLT-VLP takes advantage of this target combined with a superior delivery mechanism. There is a disadvantage of using JCPyV VLP as a gene delivery vector for clinic applications due to that most individuals are seropositive to JCPyV. Modification of the JCPyV VLP to avoid immune elimination might help this gene delivery strategy move forward to the clinic. However, further research on this system is needed to bring it closer clinical application.

Acknowledgements

This work was supported by research Grants from the National Science Council, NSC 101-2320-B-194-002-MY3 and Chia-Yi Christian Hospital, R101-11, Taiwan, ROC.

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