Antiviral Research 81 (2009) 184–187

Contents lists available at ScienceDirect

Antiviral Research

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

Short communication

Validation of BKV large T-antigen ATP-binding site as a target for drug discovery☆,☆☆

Gang Zeng[a], Marta Bueno[b], Carlos J. Camachos[b], Bala Ramaswami[a], Chunqing Luo[a], Parmjeet Randhawa[a,*]

[a] Department of Pathology, University of Pittsburgh, Pittsburgh, PA, USA
[b] Department of Computational Biology, University of Pittsburgh, Pittsburgh, PA, USA

ARTICLE INFO

Article history:
Received 1 August 2008
Received in revised form 8 November 2008
Accepted 11 November 2008

Keywords:
BK polyomavirus
Large T antigen
Drug discovery
ATP binding site
Treatment

ABSTRACT

BK virus large T antigen (LTA) is a hexameric protein with a helicase activity that is powered by ATP hydrolysis. A mutant virus with Lys420Ala, Arg421Ala, and Asp504Ala mutations at the ATP binding sites showed marked reduction in viral fitness. This observation indicates that high throughput screening for ATPase inhibitors will be valid strategy to discover anti-BKV drugs. Pilot screening of 300 compounds from the Tim Tec ActiTarg K library identified a compound, STO18584, with selectivity index of 19.2.

Polyomavirus BK (BKV) belongs to the family Polyomaviridae. Virions are 45 nm in diameter and the viral genome is arranged in three general regions: non-coding control region (NCCR), the early coding region (coding for the small and large T antigens), and the late coding region (coding for the viral capsid proteins VP-1, VP-2, VP-3, and agnoprotein) (Demeter, 1995; Shah, 1995). BKV was first isolated in 1970, and subsequent studies documented a worldwide seroprevalence rate of 60–80% (Gardner et al., 1971; Andrews et al., 1983; Gardner et al., 1984; Andrews et al., 1988). Polyomavirus reactivation with urinary shedding of infected urothelial cells is recognized to occur in 10–60% of renal transplant recipients. Viremia occurs in 5–30%, while 1–10% develop nephropathy, which results in significant graft dysfunction and may progress to graft loss. It is now recognized that patients with liver and heart transplantation or AIDS have rates of BK viruria comparable to kidney transplant patients (Josephson et al., 2003; Razonable et al., 2004; Munoz et al., 2005). BKV is also commonly excreted in the urine of bone marrow transplant recipients, in whom it is associated with mild forms of hemorrhagic cystitis in up to 60% of patients, while 5–10% develop severe hematuria. BKV associated hemorrhagic cystitis can also occur in 5% of oncology patients on who receive cyclophosphamide without routine prophylaxis (Cheerva et al., 2007). Currently, clinical management of BKV infection consists primarily of reducing immunosuppression. No drugs with proven anti-viral efficacy are currently available, although Cidofovir, Leflunomide, and FK778 have been used empirically (Scantlebury et al., 2002; Josephson et al., 2003; Farasati et al., 2005).

With a view to developing anti-BKV compounds we evaluated the large T antigen (LTA) as a potential target site, since the virus devotes almost one half of its genetic machinery to code for this protein. Theoretically, LTA is good target for drug discovery because (a) it is a key viral protein required for DNA replication, (b) it is well conserved across multiple viral strains, and (c) there is no homologous protein present in human cells, which offers of the prospect of developing anti-viral compounds with an acceptable clinical toxicity profile. LTA directs the initiation of DNA replication by assembly into a double hexameric helicase which unwinds the duplex DNA bidirectionally. The initial step is a binding of LTA to the origin binding domain in the non-coding control region (Gomez-Lorenzo et al., 2003; Li et al., 2003; Gai et al., 2004a,b). The progression of viral replication requires the recruitment of several cellular factors including human replication protein A (hRPA), DNA polymerase alpha-primase, and DNA polymerase delta (Arunkumar et al., 2005). These biochemical changes are energy dependent, and an ATPase

☆ Supported by grants RO1 AI 51227 and AI 63360 and NIH contract N01-AI 30044 to PR.

☆☆ The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health, or the National Institute of Allergy and Infectious Disease.

* Corresponding author at: Department of Pathology, Division of Transplant Pathology, University of Pittsburgh, E737 UPMC-Montefiore Hospital, 3459 Fifth Ave., Pittsburgh, PA 15213, USA. Tel.: +1 412 647 7646; fax: +1 412 647 5237.
E-mail address: randhawapa@msx.upmc.edu (P. Randhawa).

0166-3542/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2008.11.004

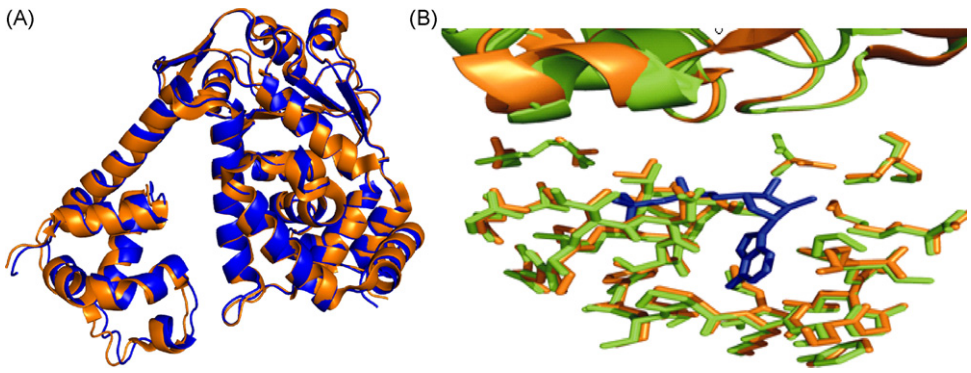

Fig. 1. (A) Three-dimensional homology model of BKV helicase domain using the SV40 crystal structure as a template. The BKV model (Swiss-Prot code: P03071) was generated using MODELLER9v1 (Marti-Renom et al., 2000) and SV40 helicase crystal structure as the template (PDB code: 1svl). Note the close spatial overlap between the SV40 protein and its BKV homolog shown in different colors (blue and orange). (B) Detailed view of the ATP binding site. The BKV helicase domain overlaps extensively with the SV40 helicase domain (green and orange colors) in the area of interaction with the ATP molecule (blue). Unlike the nucleotide base that only interacts with residues from the same monomer (termed *cis*-monomer), the phosphate and pentose moiety of ATP/ADP interact with both the *cis*-monomer and its immediate neighbor (termed *trans*-monomer). Here, the *cis*-monomer residues are depicted as sticks while the immediate monomer, or *trans*-monomer, is showed using a cartoon representation to emphasize the secondary structure (Images generated using PyMol v.0.99, DeLano Scientific LLC).

domain is present in the LTA protein (Wu et al., 2001; Gai et al., 2004a,b). Phosphorylation sites have also been described at both the N-terminal and C-terminal ends of the amino acid sequence, and can mediate activation or inactivation of viral DNA replication (Wun-Kim and Simmons, 1990; Roy et al., 2003). This large body of data led us to direct our attention to the LTA ATP binding site as a potential target for drug development.

Rational design of anti-viral drugs requires knowledge of the crystallographic structure of the target protein. A crystal structure for LTA bound to ATP is currently available only for the polyomavirus SV40 T-antigen. While BKV and SV40 show an overall DNA homology of approximately 70%, portions of the viral genome show greater divergence. Thus, the homology is only about 45% in the C-terminal portion of the LTA, encompassing amino acids 640–661(Nakshatri et al., 1988). To specifically examine the extent of homology at the ATP binding site, 13 SV40 and 30 BKV LTA sequences available in the Swiss-Prot database (Apweiler et al., 2004) were aligned using ClustalX (Chenna et al., 2003) and analyzed using BioEdit (Hall, 1999). Sequences relevant for ATP binding showed 73% amino acid identity and 90% homology, as judged by sequence alignments (based on the helicase domain of SV40 LTA, amino acids 267–627, Swiss-Prot P03070). A three dimensional homology model (Fig. 1) created with the MODELLER9v1 program using the co-crystal structure of SV40 LTA helicase domain with ADP as template (PDB code 1sv1) showed close overlap between the BK and SV40 LTA proteins (Marti-Renom et al., 2000; Gai et al., 2004b). The overlap was confirmed using six different molecular configurations, consisting of both the monomeric and dimeric forms of LTA evaluated in the free state, and bound to ATP or ADP.

An in vitro mutagenesis approach was then conceived to obtain direct proof that the ATP binding site in BKV LTA is critical for viral replication. Alignment of BKV and SV40 sequences showed that all *cis* and *trans* ATP binding residues identified as being critical in SV40 LTA are also present in BKV LTA (Table 1 and Fig. 2). The QuikChange multi site-directed mutagenesis kit (Stratagene) was used to generate mutations in pBKV 34-2 plasmid (ATCC #45025) following the manufacturer's instructions. Primers were designed to introduce Lys420Ala, Arg421Ala, and Asp504Ala mutations in the Large T-antigen ATP binding site. The three dimensional homology model referred to earlier suggests that (a) BKV Lys420 would stabilize a transition state that is critical in ATP hydrolysis and helicase function, and (b) Asp504 would to play a critical role in transferring the energy of ATP hydrolysis to DNA unwinding (Greenleaf et al., 2008). The primer sequences used for mutagenesis were: ACTGTATTGTTTTCAATGTACCTGCAGCAAGATACTGGTTATTTAAAGGTCC and GACAGTTTGAGAGATTATTTAGCTGGAAGTGTTAAGG for residues 420/421 and 504, respectively. Mutations were confirmed by whole genome BKV sequencing using our published primers (Sharma et al., 2006). No undesired mutations were seen in any other part of the viral genome. Wild type (WT) and mutant (Mu) plasmids were digested with 3 U BamHI/μg DNA for 1 h at 37 °C and transfected into Vero cells by using Lipofectamine and Lipofectamine Plus reagents as described in the manufacturer's instructions (Invitrogen, Carlsbad, CA). Viral DNA replication in Vero cells was measured in 100 mm diameter dishes 2, 4, and 6 weeks following transfection of 1 μg viral DNA (corresponding to 2E+10 copies of the 5-kb viral genome) with a well established real-time PCR assay that uses primers directed at the VP-1 gene (Randhawa et al., 2005). Viral genomic copy numbers harvested at 2, 4, and 6 week post-transfection for mutant BKV DNA were 2.03E+10 ± 8.19E+08, 5.90E+10 ± 2.9E+10, and 9.93E+10 ± 3.32E+09, respectively (mean ± s.d., $n = 3$). The corresponding figures for transfected wild type DNA were approximately 1 log higher at 2 weeks (1.29E+11 ± 1.23E+10), 2 logs higher at 4 weeks (7.72E+12 ± 2.16E+11) and 3 logs higher at 6 weeks (8.36E+13 ± 3.6E+12) ($p < 0.001$). The transfected Vero cells were

Table 1
Sequence homology between BKV and SV40 LTA at ATP binding sites.

SV40	BKV	Interactions of *cis*-residues	SV40	BKV	Interactions of *trans*-residues
K432	K434	Interact with γ-P_i and β-P_i	R540	R542	Binds γ-P_i directly
T433	T435	Coordinates an Mg^{2+} ion to interact with the γ-P_i and β-P_i	D502	D504	Interact with the γ-P_i and β-P_i through a water molecule
T434	T436	Interact directly with γ-P_i	K418	K420	Form 3 bonds with γ-P_i, α-P_i, and γ-β-oxygen
D474	D476	Interact with γ-P_i directly through hydrogen bonds	K419	R421	Interact with ribose
N529	N531	Interact with γ-P_i indirectly through a water molecule	R498	R500	Interact with ATP through a water molecule

Key residues of the large T antigen ATP/ADP binding site. The SV40 residues interacting with the ATP are extracted from Gai et al. (2004b). Homologue residues in BKV are extracted from the alignment summarized in Fig. 2.

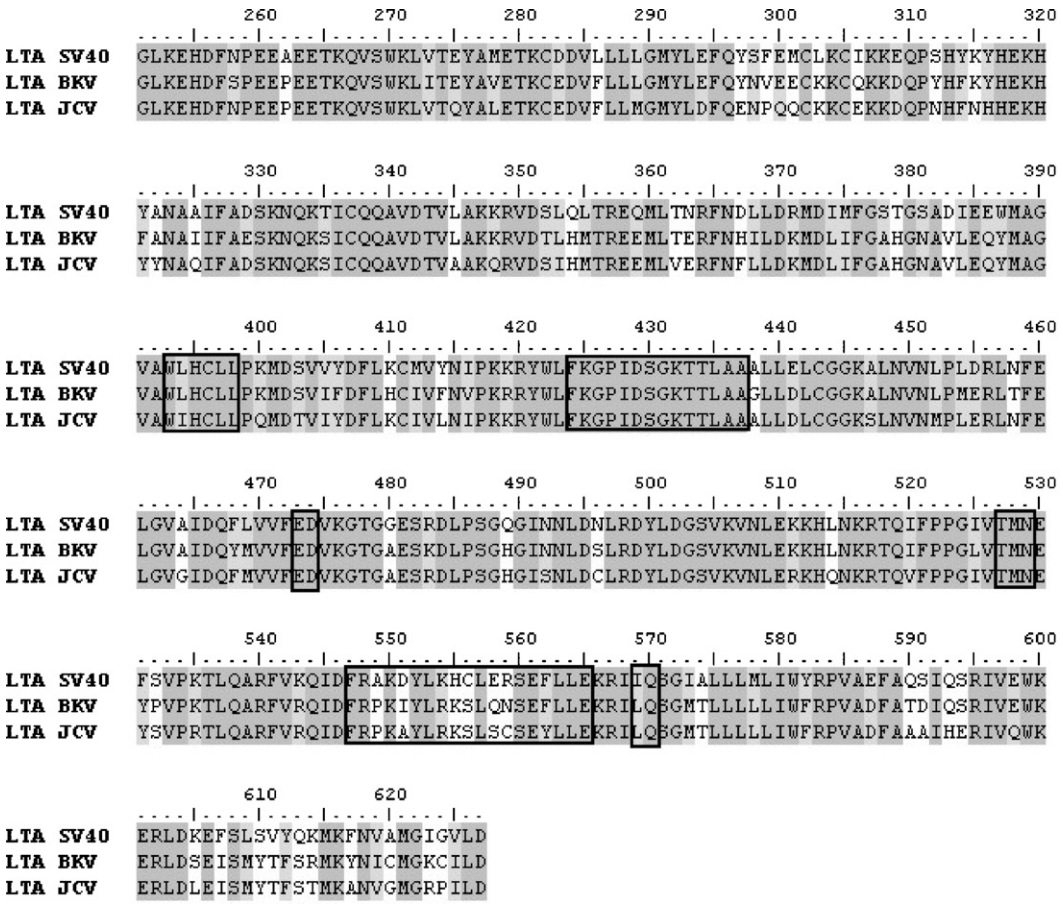

Fig. 2. Protein sequence alignment of BK, JC and SV40 LTA helicase domain (aminoacids 251–627 as in SV40 sequence). The sequences correspond to Swiss-Prot codes: P03070 (LTA SV40), P03071 (LTA BKV) and P03072 (LTA JCV). The alignment was perform using ClustalX (Chenna et al., 2003) and analyzed with BioEdit (Hall, 1999). Identical aminoacids are colored in dark grey and similar aminoacids (as determined by BLOSUM62 matrix are shown in light gray. Residues interacting with the ATP are boxed (Henikoff and Henikoff, 1992; Gai et al., 2004b).

lysed at 6 weeks and subjected to 3 freeze thaws to obtain infectious mutant or wild type BK virions. Infection experiments with these complete virions using Vero cells confirmed the marked reduction in viral fitness (replicative capacity) in mutant compared to wild type virus. Over a 6-week period of incubation, mutant virus multiplied less than 10-fold compared to approximately one million-fold for wild type virus (Fig. 3). As a direct test of the validity of the ATP

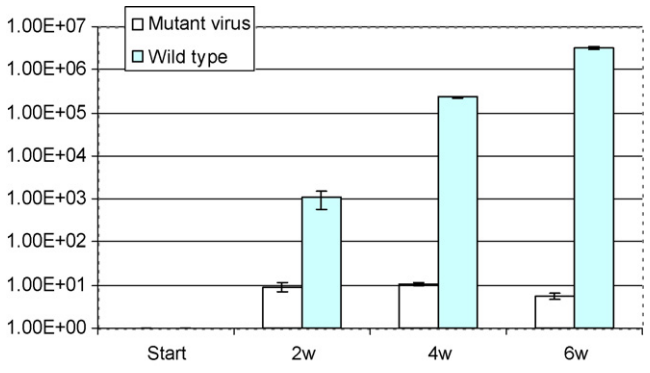

Fig. 3. Total viral yield in genomic copies for wild type (colored bar) and mutant virus (colorless bar) measured in cell pellets 2, 4, and 6 weeks after culture (*X*-axis), and expressed as fold change with respect to day 1 (*Y*-axis). Data represents results of real time PCR experiments in which 1.8E+06 Vero cells grown in a 10-cm dish were infected with 1.8E+08 BKV particles (multiplicity of infection 100:1), and harvested at the indicated time points (mean ± s.d., $n = 3$, $p < 0.001$).

binding site as a therapeutic target, we have tested 300 compounds from the TimTec ActiTargK Library of kinase/ATPase inhibitors using a previously published real time PCR drug sensitivity assay (Randhawa et al., 2006). One compound, STO18584 is active, as judged by a CC50 of 21.09 ± 2.32 μM, EC50 of 1.1 ± 0.39 μM, and a SI = 19.2.

In conclusion, we have shown that BKV LTA ATP binding site contains amino acids that play an important part in mediating replication of viral DNA. Hence, drugs inhibiting LTA ATPase activity would have anti-viral activity that could be exploited for the treatment of BKV infection in the clinical arena. Sequence alignments show conservation of these critical BKV amino acids in polyomavirus JC virus (JCV) and SV40 (Fig. 2). Hence, any anti-BKV compounds discovered by initiating a drug discovery program for ATPase inhibitors will likely also be useful to treat JCV and SV40 infections in man. There are increasing reports of JCV infection leading to viral nephropathy, and one recent study estimates the prevalence of this complication in kidney transplant patients to be 0.9% (Kazory et al., 2003; Drachenberg et al., 2007). Additionally, JCV is associated progressive multifocal encephalopathy in patients with AIDS. Clinical syndromes associated with replicative SV40 infection, including occasional cases of nephropathy, have only rarely been described in man (Milstone et al., 2004), although there is more abundant, albeit somewhat controversial, evidence that latent SV40 infection is associated with neoplasms such as mesothelioma, osteogenic sarcoma, and lymphoma (Butel, 2001; Vilchez et al., 2002).

References

Andrews, C.A., Daniel, R.W., et al., 1983. Serologic studies of papovavirus infections in pregnant women and renal transplant recipients. Prog. Clin. Biol. Res. 105, 133–141.

Andrews, C.A., Shah, K.V., et al., 1988. A serological investigation of BK virus and JC virus infections in recipients of renal allografts. J. Infect. Dis. 158 (1), 176–181.

Apweiler, R., Bairoch, A., et al., 2004. Protein sequence databases. Curr. Opin. Chem. Biol. 8 (1), 76–80.

Arunkumar, A.I., Klimovich, V., et al., 2005. Insights into hRPA32 C-terminal domain-mediated assembly of the simian virus 40 replisome. Nat. Struct. Mol. Biol. 12 (4), 332–339.

Butel, J.S., 2001. Increasing evidence for involvement of SV40 in human cancer. Dis. Markers 17 (3), 167–172.

Cheerva, A.C., Raj, A., et al., 2007. BK virus-associated hemorrhagic cystitis in pediatric cancer patients receiving high-dose cyclophosphamide. J. Pediatr. Hematol. Oncol. 29 (9), 617–621.

Chenna, R., Sugawara, H., et al., 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 31 (13), 3497–3500.

Demeter, L.M., 1995. JC, BK, and other polyomaviruses; progressive multifocal leukoencephalopathy. In: Mandel, G.L., Bennett, J.E., Dolin, R. (Eds.), Principles and practice of infectious diseases. Churchill Livingstone, New York, pp. 1400–1406.

Drachenberg, C.B., Hirsch, H.H., et al., 2007. Polyomavirus BK versus JC replication and nephropathy in renal transplant recipients: a prospective evaluation. Transplantation 84 (3), 323–330.

Farasati, N.A., Shapiro, R., et al., 2005. Effect of leflunomide and cidofovir on replication of BK-virus in an in vitro culture system. Transplantation 79 (1), 116–118.

Gai, D.H., Li, D.W., et al., 2004a. Insights into the oligomeric states, conformational changes, and helicase activities of SV40 large tumor antigen. J. Biol. Chem. 279 (37), 38952–38959.

Gai, D.H., Zhao, R., et al., 2004b. Mechanisms of conformational change for a replicative hexameric helicase of SV40 large tumor antigen. Cell 119 (1), 47–60.

Gardner, S.D., Field, A.M., et al., 1971. New human papovavirus (B.K.) isolated from urine after renal transplantation. Lancet 1 (7712), 1253–1257.

Gardner, S.D., MacKenzie, E.F., et al., 1984. Prospective study of the human polyomaviruses BK and JC and cytomegalovirus in renal transplant recipients. J. Clin. Pathol. 37 (5), 578–586.

Gomez-Lorenzo, M.G., Valle, M., et al., 2003. Large T antigen on the simian virus 40 origin of replication: a 3D snapshot prior to DNA replication. EMBO J. 22 (23), 6205–6213.

Greenleaf, W.B., Shen, J., et al., 2008. Systematic study of the functions for the residues around the nucleotide pocket in simian virus 40 AAA+ hexameric helicase. J. Virol. 82 (12), 6017–6023.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. (41), 95–98.

Henikoff, S., Henikoff, J.G., 1992. Amino acid substitution matrices from protein blocks. Proc. Natl. Acad. Sci. U.S.A. 89 (22), 10915–10919.

Josephson, M.A., Poduval, R.D., et al., 2003. BK nephropathy: can leflunomide control it? J. Am. Soc. Nephrol. 14, 43A–143A.

Kazory, A., Ducloux, D., et al., 2003. The first case of JC virus allograft nephropathy. Transplantation 76 (11), 1653–1655.

Li, D.W., Zhao, R., et al., 2003. Structure of the replicative helicase of the oncoprotein SV40 large tumour antigen (vol. 423, p. 512, 2003). Nature 425 (6953), 102–1102.

Marti-Renom, M.A., Stuart, A.C., et al., 2000. Comparative protein structure modeling of genes and genomes. Annu. Rev. Biophys. Biomol. Struct. 29, 291–325.

Milstone, A., Vilchez, R.A., et al., 2004. Polyomavirus simian virus 40 infection associated with nephropathy in a lung-transplant recipient. Transplantation 77 (7), 1019–1024.

Munoz, P., Fogeda, M., et al., 2005. Prevalence of BK virus replication among recipients of solid organ transplants. Clin. Infect. Dis. 41 (12), 1720–1725.

Nakshatri, H., Pater, M.M., et al., 1988. Functional role of BK virus tumor antigens in transformation. J. Virol. 62 (12), 4613–4621.

Randhawa, P., Farasati, N.A., et al., 2006. Ether lipid ester derivatives of cidofovir inhibit polyomavirus BK replication in vitro. Antimicrob. Agents Chemother. 50 (4), 1564–1566.

Randhawa, P., Vats, A., et al., 2005. Monitoring for polyomavirus BK and JC in urine: comparison of quantitative polymerase chain reaction with urine cytology. Transplantation 79 (8), 984–986.

Razonable, R.R., Brown, R.A., et al., 2004. Human polyomavirus infection is common in cytomegalovirus (CMV) D+/R− solid organ transplant (SOT) patients. Am. J. Transplant. 4, 338–339.

Roy, R., Trowbridge, P., et al., 2003. The cap region of topoisomerase I binds to sites near both ends of simian virus 40 T antigen (vol. 77, p. 9809, 2003). J. Virol. 77 (20), 11301–111301.

Scantlebury, V., Shapiro, R., et al., 2002. Cidofovir: a method of treatment for BK virus associated transplant nephropathy. Graft 5 (supplement), S82–S86.

Shah, K.V., 1995. Polyomaviruses. In: Fields, B.N., Knipe, M.K., Howley, P.M. (Eds.), Virology. Lippincott-Raven, Philadelphia, pp. 2027–2043.

Sharma, P.M., Gupta, G., et al., 2006. A phylogenetic analysis of polyomavirus BK sequences. J. Virol. 80, 8869–8879.

Vilchez, R.A., Madden, C.R., et al., 2002. Association between simian virus 40 and non-Hodgkin lymphoma. Lancet 359 (9309), 817–823.

Wu, C.X., Roy, R., et al., 2001. Role of single-stranded DNA binding activity of T antigen in simian virus 40 DNA replication. J. Virol. 75 (6), 2839–2847.

Wun-Kim, K., Simmons, D.T., 1990. Mapping of helicase and helicase substrate-binding domains on simian virus 40 Large T antigen. J. Virol. 64 (5), 2014–2020.