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Research Paper

Preparation and application of polyclonal antibodies against KSHV v-cyclin

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Abstract

We prepared rabbit polyclonal antibodies against Kaposi´s sarcoma-associated herpesvirus (KSHV)-encoded v-cyclin (ORF 72) and detected the natural viral protein using these polyclonal antibodies. Three antigenic polypeptides of v-cyclin were designed and synthesized. A fragment of the v-cyclin gene was cloned into a eukaryotic expression vector pEF-MCS-Flag-IRES/Puro to construct a recombinant vector, pEF v-cyclin. Then, pEF v-cyclin was transfected into 293T and EA.hy926 cells to obtain v-cyclin-Flag fusion proteins. Six New Zealand white rabbits were immunized with KLH-conjugated peptides to generate polyclonal antibodies against v-cyclin. The polyclonal antibodies were then characterized by ELISA and Western blotting assays. Finally, the polyclonal antibodies against v-cyclin were used to detect natural viral protein expressed in BCBL-1, BC-3, and JSC-1 cells. The results showed that using the Flag antibody, v-cyclin-Flag fusion protein was detected in 293T and EA.hy926 cells transfected with pEF-v-cyclin. Furthermore, ELISA showed that the titer of the induced polyclonal rabbit anti-v-cyclin antibodies was higher than 1:8,000. In Western blotting assays, the antibodies reacted specifically with the v-cyclin-Flag fusion protein as well as the natural viral protein. The recombinant expression vector pEF-v-cyclin was constructed successfully, and the polyclonal antibodies prepared can be used for various biological tests including ELISA and Western blotting assays.

Keywords: Kaposi´s sarcoma-associated herpesvirus, v-cyclin, synthesized peptides, polyclonal antibody

INTRODUCTION

Kaposi´s sarcoma-associated herpesvirus (KSHV, also known as human herpesvirus 8, HHV-8) is a γ-2-herpesvirus associated with three human malignancies, Kaposi´s sarcoma, primary effusion lymphoma, and multicentric Castleman´s Disease[1-3]. Kaposi´s sarcoma (KS) lesions are characterized by proliferating spindle cells, prominent angiogenesis, hemorrhage, and leukocyte infiltration. The *KSHV* genome encodes more than 90 open reading frames (ORFs) and 25 mature miRNAs[4]; many of them possess oncogenic properties[5][6-8]. Among them, 15 proteins are unique to KSHV and four KSHV pro–

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teins: kaposin (encoded by ORF K12)[9], v-FLIP (ORF 71/ K13)[10], v-cyclin (ORF 72), and the latency-associated nuclear antigen (ORF 73/LANA)[11], are detected consistently in all latently infected cells. It has been demonstrated that these gene products promote cellular proliferation and cellular survival, prevent apoptosis, facilitate immune evasion, and maintain the extrachromosomal viral genome during repeated cell divisions[12-15]. Each of these functions is likely to be important in KSHV pathogenesis[16-17], especially KSHV v-cyclin, which modulates the cell cycle by phosphorylating p27. In primary effusion lymphoma cells, the v-cyclin Cdk6 complex phosphorylates p27KIP1, which is highly expressed in primary effusion lymphoma cell lines, inducing its degradation via a proteasome-dependent pathway. This function has been implicated in the development of KS tumors and the induction of lymphomas[18-20].

In this study, we designed three v-cyclin polypeptides according to a bioinformatics software analysis. To explore the biological function of v-cyclin, a fragment of the v-cyclin gene from pCDH v-cyclin was cloned into a eukaryotic expression vector pEF-MCS-Flag-IRES/Puro to construct a recombinant pEF-v-cyclin vector. By immunizing New Zealand white rabbits with v-cyclin-KLH, we generated polyclonal antibodies against KSHV v-cyclin (the peptides were conjugated to keyhole limpet hemocyanin (KLH) to increase antigenicity). The antibodies prepared against v-cyclin were shown to be useful for detecting the expression of v-cyclin in transfected cells and natural viral protein expressed in (KSHV$^+$) BCBL-1, BC-3 PEL, and KSHV$^+$ EBV$^+$ JSC-1 PEL cells. The antibodies will be helpful in further studies of the role of v-cyclin in KSHV infection and KS pathology.

MATERIALS AND METHODS

Animals, cells, plasmids, and transfection

Six Male New Zealand white rabbits (6 weeks old, female, 3 kg) were purchased from BaiQi Biotechnology (Suzhou, China). HEK 293T (human embryonic kidney) cells were cultured as described previously[21,22]. EA.hy926, KSHV$^+$ BCBL-1, BC-3 PEL, and KSHV$^+$ EBV$^+$ JSC-1 PEL cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L Lglutamine, and antibiotics. The pEF-MCS-Flag-IRES/Puro and pCDH-v-Cyclin plasmids were provided by Dr. Shou-Jiang Gao (University of Southern California). Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer´s protocol.

Construction of the expression plasmid pEF-v-cyclin Flag-IRES/Puro (pEF v-cyclin)

The full-length cDNA of KSHV v-cyclin (NCBI Reference Sequence: YP_001129430.1) consists of 771 base pairs (bp), encoding a 257 amino acid protein. The full extracellular fragment of KSHV v-cyclin was amplified by the polymerase chain reaction (PCR) from pCDH-v-cyclin using the primers 5´-TCT*CTCGAG*TTAATAGCTGTCCAGAATGCG-CAGA-3´ (sense) and 5´-TAT*GCTAGC*GGCAACT-GCCAATAACCCGCCCTCGGGA-3´ (anti-sense). The 5´ extensions include *Xho*I and *Nhe*I restriction sites, indicated in italics. The PCR fragment comprised of the extracellular fragment of KSHV v-cyclin was inserted into the eukaryotic expression vector pEF-MCS-Flag-IRES/Puro and sequenced by a commercial service (Invitrogen, Shanghai, China).

Composition of v-cyclin polypeptide and production of polyclonal antibodies against v-cyclin

Three v-cyclin polypeptides coupled with KLH were designed using the SUMOplot software and were synthesized by BaiQi Biotechnology. Rabbit serum against KSHV v-cyclin was then obtained according to standard protocols. Briefly, six adult female New Zealand white rabbits were immunized and each rabbit was injected subcutaneously with 400 μg of purified ectopic v-cyclin-KLH polypeptides emulsified with complete or incomplete Freund´s adjuvant (Sigma). Each polypeptide was used to immunize two rabbits: RB1A and RB1B were immunized with polypeptide No. 1, RB2A and RB2B were immunized with polypeptide No. 2 and RB3A and RB3B were immunized with polypeptide No. 3. Then, each rabbit was booster-immunized with 200 μg v-cyclin-KLH polypeptides emulsified in complete or incomplete Freund´s adjuvant by subcutaneous injection once every 2 weeks. Before immunization, blood was obtained from each animal to prepare non-immune serum, and after four booster immunizations, blood was obtained again. The serum samples were stored at -80°C and the immunization efficiency was analyzed by ELISA and Western blotting.

Purification of anti-v-cyclin and determination of anti-v-cyclin titer by ELISA

Anti-v-cyclin was purified with caprylic acid-ammonium sulfate precipitation according to a reported protocol[23,24]. One volume of antiserum was diluted with four volumes of 0.06 M acetic acid buffer (17.4

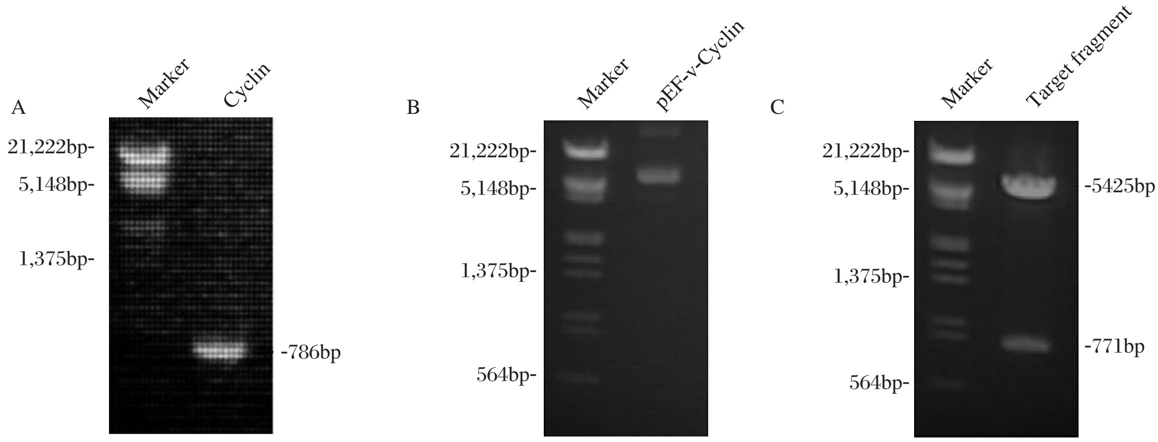

Fig 1. **Amplification of the KSHV *v-cyclin* gene and construction of recombination plasmid pEF v-cyclin Flag-IRES/Puro.** A: Amplification of the v-cyclin gene by PCR. B and C: pEF v-cyclin and fragments of pEF v-cyclin restrictly digested by *NheI* and *XhoI*.

M glacial acetic acid was diluted to 0.06 M with distilled water, and adjusted to pH 4.5 with 5 M NaOH). At room temperature, caprylic acid was added dropwise to a final concentration of 25 μL/mL. After vigorous mixing for 30 minutes, the sample was centrifuged (10,000 g, 10 minutes, 4°C) to remove floaters and the precipitate. The supernatant was filtered through filter paper three times. The solution was then adjusted to pH 7.4 and kept in an ice bath for 10 minutes. Solid ammonia/sulfuric acid (0.277 g/mL) was added to the solution. After stirring for 30 minutes at 4°C, the solution was centrifuged (10,000 *g*, 10 minutes). The supernatant was discarded and the precipitate was dissolved in PBS and dialyzed against PBS until no NH_4^+ was present.

The titers of the anti-v-cyclin antibodies were determined using an indirect enzyme-linked immunosorbent analysis (ELISA). First, wells in the 96-well immunoplates were coated with purified v-cyclin, diluted to 1 mg/L with 50 mmol/L carbonate-bicarbonate buffer (pH 9.6), 100 μL per well, at 4°C overnight and then were washed three times with washing buffer. Next, the coated wells were blocked with 100 μL 10% fetal calf serum (FCS) for 2 hours at 37°C, followed by incubation with 100 μL of anti-v-cyclin antibodies with serial dilutions of 1:2 (normal serum without immunization as negative control). After incubation for 1 hour at 37°C, the wells were washed and incubated with 100 μL HRP-conjugated goat-anti-rabbit IgG (dilution 1:5,000 Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at 37°C. Finally, the wells were washed five times with washing buffer, and then incubated with 100 μL enzyme substrate for 3 hours at 37°C. Then, color development was stopped and absorbance was measured at 450 nm using a microplate reader.

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Query    1  GGTATCCTGCGGAATGACGTTGGCAGGAACCAACAGCGCACAGCCTGCAGCGCTGATAAT  60
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Sbjct  130  GGTATCCTGCGGAATGACGTTGGCAGGAACCAACAGCGCACAGCCTGCAGCGCTGATAAT  189

Query   61  AGAGGCGGGCAATGAGCCAGTCTTTGGGTCAACTAAGGCTTTTGTAATCAGGGTGTTGAC  120
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Query  121  CTCGTGGTGCCAAAAGTCCAGGTGTTGGGAGCCCCCCAGCAATTTAAGTAACAAGAAGGA  180
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Query  181  AGTGACGTCCGTCGCTAAGACTGCCTCTGTTCGCCACGCCAACTTCTCAAGGAGTTCTTT  240
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Sbjct  310  AGTGACGTCCGTCGCTAAGACTGCCTCTGTTCGCCACGCCAACTTCTCAAGGAGTTCTTT  369

Query  241  CTCCTGGTCTATAAGTTCTTGGCGGGAAA  269
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Sbjct  370  CTCCTGGTCTATAAGTTCTTGGCGGGAAA  398
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Fig 2. **The sequence of pEF-MCS-Flag-IRES/Puro was compared with KSHV ORF 72 by DNA sequencing.** A fragment corresponding to the *v-cyclin* gene was inserted in an expression vector, pEF-MCS-Flag-IRES/Puro, after cleavage with *NheI* and *XhoI*. The correct insertion, identity, and integrity of the v-cyclin gene were demonstrated by DNA sequencing, which showed an identical sequence to that in GenBank.

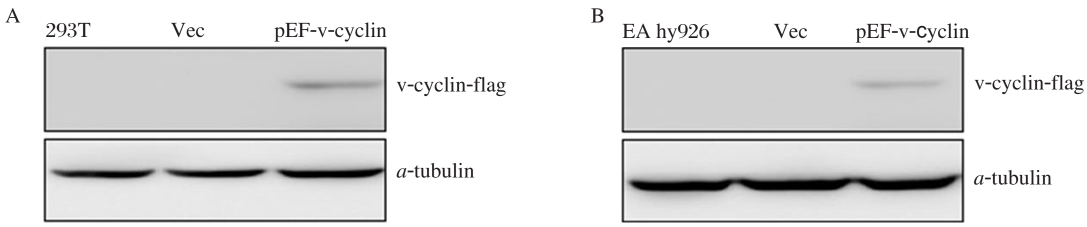

Fig 3. **Western blotting analyses using the antibody against KSHV v-cyclin-flag.** A: Expression of the v-cyclin protein in 293T cells transfected with pEF-v-cyclin was detected with the anti-flag antibody in Western blotting. B: Expression of the v-cyclin protein in EA.hy926 cells transfected with pEF-v-cyclin was detected with the anti-flag antibody in Western blotting assays.

Western blotting assays

Lysates of 293T cells transfected with pEF-v-cyclin, BCBL-1, BC-3, and JSC-1 cells were separated by 12% SDS-PAGE. Expression of the full-length v-cyclin protein was detected by Western blotting assays as described previously[25]. The primary antibody used in Western blotting assays was the prepared anti-v-cyclin antibodies.

Statistical analysis

All experiments were performed at least in triplicate unless noted otherwise. Numerical data are expressed as means±SD. Two-group comparisons were analyzed by a two-sided Student´s t-test. P values < 0.05 were considered to indicate statistical significance.

RESULTS

Amplification of the KSHV v-cyclin gene and construction of recombination plasmid pEF-v-cyclin-Flag-IRES/Puro

Amplification of the v-cyclin gene by PCR, pEF(v-cyclin) and products of pEF-v-cyclin cleaved with the restriction enzymes NheI and XhoI, were confirmed by 1.0% agarose gel (w/v) electrophoresis. v-cyclin with an expected size of 786 bp was detected by agarose gel electrophoresis (***Fig. 1A***). The recombination expression plasmid, pEF-v-cyclin-Flag-IRES/Puro, and the products cleaved by the restriction enzymes *Nhe*I and *Xho*I, were both detected by agarose gel

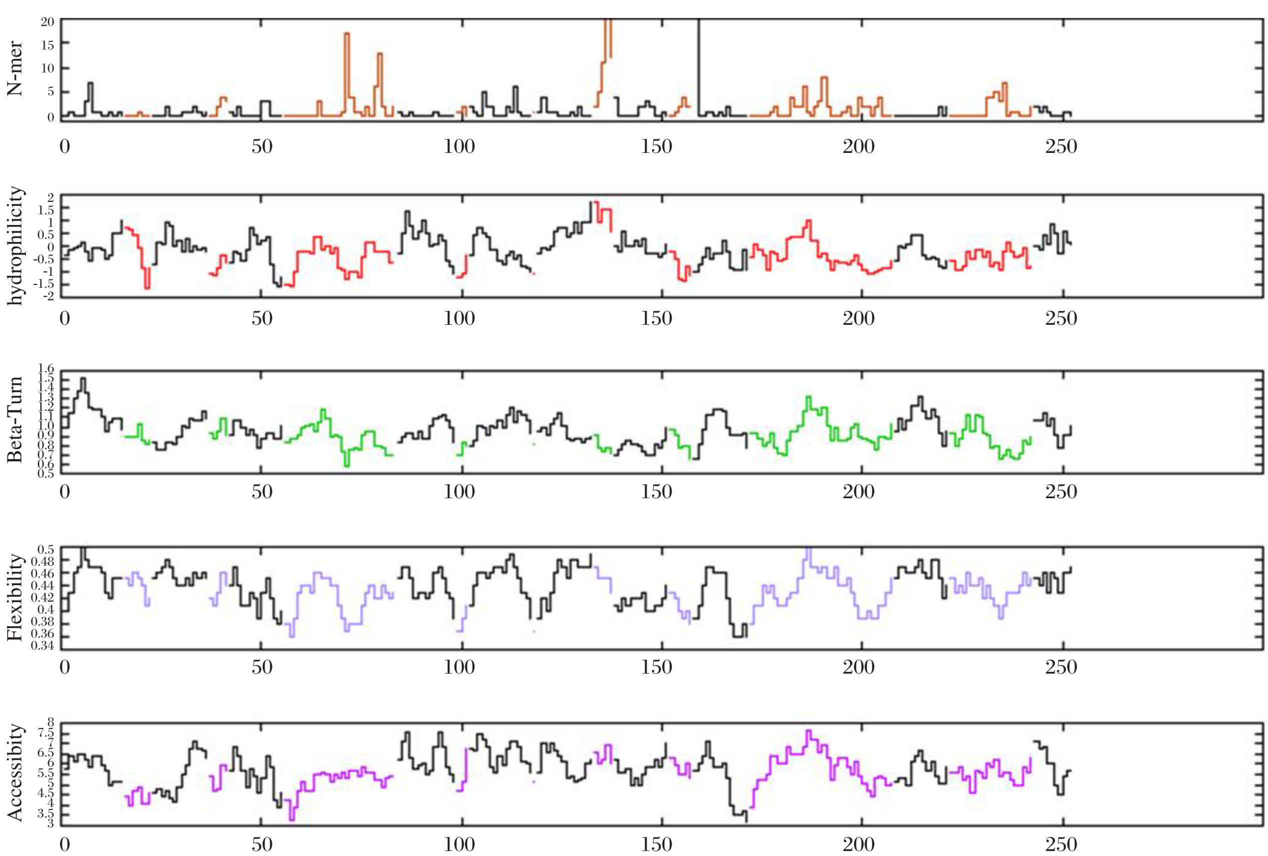

Fig 4. **Protein profiles of v-cyclin analyzed using the SUMOplot software.**

Table 1 **Epitope candidates designed for v-cyclin**

No.	Epitope	Len	Start	End	Score (1-5)	Rabbit No.
1	NH2-CSVSDFDLRILDSY-COOH	14	244	257	3.4	RB1A-B
2	NH2-TKALVDPKTGSLC-CONH2	12	181	192	3.4	RB2A-B
3	NH2- SLTSHMRKLLGC-CONH2	11	45	55	1.8	RB3A-B

electrophoresis. All showed the expected size (***Fig. 1B*** and ***1C***). The correct insertion, identity, and integrity of the *v-cyclin* gene were demonstrated by DNA sequencing, which showed an identical sequence compared with KSHV ORF 72 in GenBank (accession number YP_001129430.1; ***Fig. 2***).

Expression of v-cyclin in 293T and EA.hy926 cells

First, 293T and EA.hy926 cells were transfected with pEF-v-cyclin. Then, expression of v-cyclin was detected by Western blotting assays. An anti-Flag antibody was used as the primary antibody in this detection. Lysates of both cell types were separated by 12% SDS-PAGE. V-cyclin with an expected molecular weight of 28 kDa was detected clearly in the insoluble fraction of cell lysates (***Fig. 3***).

Preparation, purification, and identification of v-cyclin-KLH fusion protein

According to a series of parameters, such as antigen hydrophilicity, stable conformation, and a linear epitope (***Fig. 4***), three antigenic polypeptides of KSHV v-cyclin were designed and synthesized (***Table 1***). At the same time, v-cyclin was prepared and purified according to a routine protocol. Next, six adult female New Zealand white rabbits were immunized with purified v-cyclin-KLH polypeptides to generate polyclonal antibodies against the extracellular fragment of v-cyclin (anti-v-cyclin antibodies). Then, anti-v-cyclin IgG was purified with caprylic acid-ammonium sulfate precipitation according to a routine protocol.

Determining the titer of v-cyclin antisera by ELISA

Rabbits were immunized with v-cyclin-KLH to generate polyclonal antibodies against the extracellular region of v-cyclin. The purification of anti-v-cyclin antibodies was carried out by affinity chromatography. Positive sera containing anti-v-cyclin antibodies and negative serum collected before immunization were diluted (from 1:1,000 to 1:8,000) and their reactivity with v-cyclin was determined by ELISA (***Table 2***). The titer of anti-v-cyclin serum was determined to be 1:8,000.

Detection of full-length v-cyclin protein with anti-v-cyclin antibodies by Western blotting

293T cells were transfected with pEF-v-cyclin encoding the full-length v-cyclin. Whole lysates of transfected 293T cells were subjected to SDS-PAGE and the full-length v-cyclin protein was clearly detected with anti-v-cyclin antibodies from RB1A, RB1B (rabbits immunized with polypeptide No. 1) and RB2A and RB2B (rabbits immunized with polypeptide No. 2) on a Western blot (***Fig. 5***). To assess detection of natural KSHV v-cyclin by anti-v-cyclin antibody, lysates of ($KSHV^+$) BC-3, BCBL-1 PEL, and ($KSHV^+$ EBV^+) JSC-1 PEL cells were subjected to SDS-PAGE. However, only anti-v-cyclin antibodies from RB2B clearly detected the full-length v-cyclin protein with the predicted molecular weight on a Western blot. Western blotting results showed that the expression level of v-cyclin in JSC-1 cells was clearly higher than that in BC-3 and BCBL-1 cells without TPA treatment and that expression of KSHV latent v-cyclin increased in BCBL-1, BC-3, and JSC-1 cells after 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment (***Fig. 6***).

DISCUSSION

KSHV encodes some proteins with homology to cellular proteins, such as cyclin D, G-protein-coupled protein, interleukin-6, and macrophage inflammatory proteins-1 and -2. These viral proteins can mimic or disrupt host cytokine signaling in microenviron–

Table 2 **Detection of antibody titer in serum by indirect ELISA**

No.	Antigen	Blank	Negative 1:1000	Negative 1:4000	Positive 1:1000	Positive 1:4000	Positive 1:8000
RB1A	1	0.105	0.13	0.085	2.948	1.257	0.686
RB1B	1	0.101	0.099	0.091	2.759	1.249	0.709
RB2A	2	0.082	0.15	0.099	3.628	3.191	2.544
RB2B	2	0.073	0.203	0.122	3.234	3.146	2.674
RB3A	3	0.08	0.163	0.108	3.551	2.732	1.681
RB3B	3	0.084	0.163	0.107	2.219	2.741	0.473

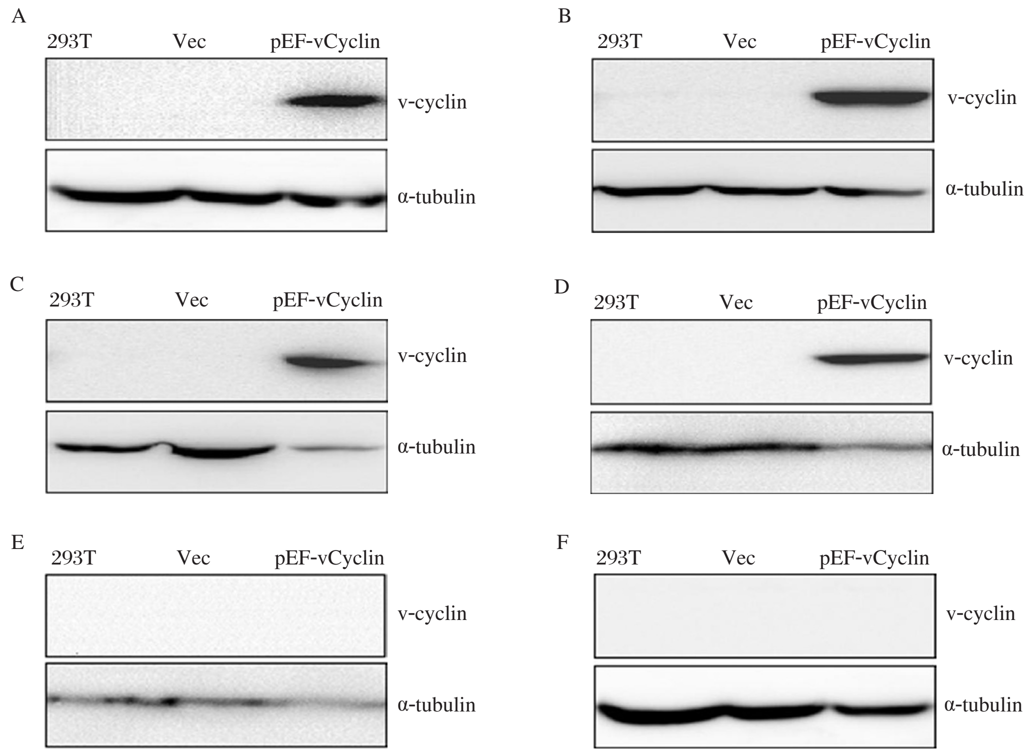

***Fig 5.* Detection of the full-length v-cyclin protein by anti-v-cyclin in Western blotting assays.** A and B: The KSHV v-cyclin protein (predicted MW of 28 kDa) was detected with the anti-v-cyclin antibodies from RB1A, RB1B (rabbits immunized with polypeptide No. 1); C and D: The KSHV v-cyclin protein was also detected with the anti-v-cyclin antibodies from RB2A, RB2B (rabbits immunized with polypeptide No. 2); E and F: The KSHV v-cyclin protein was also detected with the anti-v-cyclin antibodies from RB3A and RB3B (rabbits immunized with polypeptide No. 3).

ments, resulting in tumor growth. Latent infection is predominant in KSHV infection and KSHV encodes a latency-associated gene cluster, including ORF73/LANA-1, v-cyclin (ORF72), viral FLICE-inhibitory protein (K13, v-FLIP), kaposin (K12), and viral-encoded miRNAs. In particular, the KSHV v-cyclin gene encodes a 257-amino-acid protein with a molecular weight of 28 kDa. The v-cyclin protein has 32% identity and 54% similarity to mammalian cyclin D2, with the homology extending over the full length of the protein[26]. *V-cyclin* is a latent KSHV gene that is transcribed from the same promoter element as LANA (encoded by ORF 73). ORF 71, 72 and 73 belong to a multicistronic transcriptional unit, known as the latency transcript (LT) cluster. It is likely that LANA is the principal translation product of the longer mRNA, whereas both v-cyclin and v-FLIP (encoded by ORF 71) are synthesized from the shorter transcript, the latter by way of an internal ribosome entry site upstream of ORF 71. Expression of ORFs 71, 72, and 73 can be induced by TPA stimulation.

Cyclin-dependent kinase 6 (CDK6) is the catalytic subunit of the protein complex and v-cyclin can bind tightly to CDK6 to form a potent phosphorylation complex that phosphorylates many targets, including p27KIP1, Id-2, Cdc25a, histone H1, Cdc6, Orc1, and Bcl-2[10, 20,27,28]. The v-cyclin-CDK6 protein complex is resistant to cell cycle checkpoint regulation, contributing to increased rates of cell proliferation[29]. For example, the v-cyclin-CDK6 complex phosphorylates and inactivates Bcl-2, thus contributing to v-cyclin-mediated apoptosis[30]. On the other hand, the v-cyclin-CDK6 complex phosphorylates and inactivates Rb, which serves as an important cell cycle checkpoint protein by binding to the transactivation domain of E2F transcription factors, inhibiting their functions and preventing cell cycle progression[31,32]. Consequently, KSHV-infected cells can avoid Rb-induced cell cycle arrest by the v-cyclin-CDK6 complex[33,34]. p27KIP1 is another important regulator of cell cycle and v-cyclin can overcome p27KIP1-mediated cell cycle arrest[34-36]. When v-cyclin is overexpressed in cells, the v-cyclin-CDK6 complex interacts with and phosphorylates p27KIP1 at Thr187, inducing its degradation via a proteasome-dependent pathway[34,37]. v-cyclin-CDK6 forms a stable complex with p27KIP1, which is highly expressed in PEL cell lines[20,38,39], thereby inducing degradation of p27KIP1 via phosphorylation of Thr187, preventing it from carrying out its normal function[39].

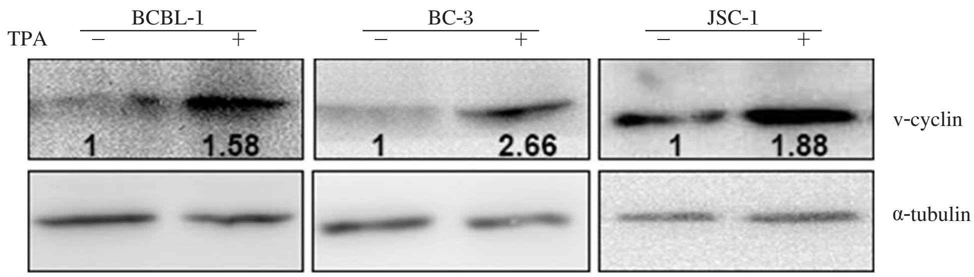

***Fig 6.* Detection of natural viral protein in BCBL-1, BC-3, and JSC-1 cells in Western blotting assays.** Lysates of KSHV+ BC-3, BCBL-1 PEL, and KSHV+ EBV+ JSC-1 PEL cells with or without TPA treatment were subjected to SDS-PAGE. The anti-v-cyclin antibody came from rabbit RB2B, immunized with polypeptide No. 2.

To ensure that synthetic peptides produce an im–mune reaction, peptides are usually designed greater than normal length. In our study, we obtained anti-v-cyclin antibodies from six rabbits immunized with three different antigen peptides coupled with KLH. Western blotting assays confirmed that the RA1A, RA1B, RA2A, and RA2B anti-v-cyclin antibod–ies clearly detected the full-length v-cyclin protein expressed in 293T cells. However, RA3A and RA3b antibodies did not identify the recombinant v-cyclin protein. This might be attributable to the correspond–ing epitope identified by polyclonal antibodies RA3A and RA3b not being present at the surface of the protein. The anti-protein antibody cannot bind when the main epitope is not exposed because of protein folding[40,41]. Another possible explanation is that the FLAG-tag impacted the three dimensional conforma–tion of the recombinant protein.

To find the most useful antibody for clinical ap–plications, we evaluated the immunoreactivities of the four kinds of polyclonal antibodies in recognizing natural KSHV v-cyclin protein[42]. Using the antibod–ies prepared, the recombinant full-length v-cyclin protein was clearly detected with the polyclonal an–tibodies from RB1A, RB1B, RB2A, and RB2B, but only the anti-v-cyclin antibody from RB2B generated in a rabbit immunized with the peptide antigen No. 2 clearly detected the natural protein in BCBL-1, BC-3, and JSC-1 cells. A possible explanation is differences in membrane circumstances, including lipid compo–nents and/or posttranslational modification between the recombinant systems and human tissues. The activities of ectopic v-cyclin protein do not directly and quantitatively mirror the actual KSHV v-cyclin activities in human tissues. These results suggest that the specificity of the polyclonal antibodies is not controllable and not all the polyclonal antibodies will be useful for clinical diagnostic assays. Because most antigens are highly complex, they present numer–ous epitopes that are recognized by a large number of antibodies. Antibodies recognize epitopes of varying size and may bind an epitope using some or all of its six complementarity-determining regions (CDRs). Binding of an epitope to its antibody is reversible and depends on the precise antibody- antigen configura–tion. Relatively minor changes in antigen structure can markedly affect the strength of the interaction.

KSHV latent v-cyclin expression increased in BCBL-1, BC-3, and JSC-1 cells after TPA treatment. Several studies have provided evidence of epigenetic control of KSHV reactivation. One showed that TPA treatment not only caused demethylation of the RTA promoter but also induced KSHV lytic replication. TPA can also enhance KSHV lytic cycle proliferation through activa–tion of the protein kinase C (PKC) and AP-1 pathways, promoting the expression of the KSHV latency-associ–ated gene cluster, including latent v-cyclin.

Using the prepared antibodies, the normalized activi–ties of polyclonal antibodies in JSC-1 cells were unam–biguously higher than those in BC-3 and BCBL-1 cells without TPA treatment (***Fig. 6***). This may be attribut–able to differing *KSHV* gene expression in the three cell lines. Previous studies have demonstrated that JSC-1 showed higher basal and induced expression of KSHV lytic cycle gene products (viral interleukin-6[vIL-6]and viral thymidine kinase[vTK]) than did the BC-3, BCBL-1, and HBL-6 cell lines. JSC-1 cells yield supernatant virions that are highly infectious in an in vitro infection assay and viral supernatant from JSC-1 cells was much more effective in infecting primary human dermal mi–crovascular endothelial cells with KSHV than superna–tants from BC-3 and BCBL-1 PEL cell lines.

In conclusion, we have produced polyclonal an–tibodies that specifically recognize KSHV v-cyclin. The antibodies can be used in various biological tests including further studies on KSHV infection and KS pathology after affinity chromatography purification.

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