Kaposi's Sarcoma-Associated Herpesvirus Infection Promotes Proliferation of SH-SY5Y Cells by the Notch Signaling Pathway

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Abstract

Background: The cancer caused by Kaposi's sarcoma-associated herpesvirus (KSHV) is one of the major causes of death in AIDS. Some patients have neurological symptoms and may be linked with KSHV infection, based on the herpesvirus has shown a neurotropic tendency in recent years.
The objective of this study is to detect the effects of KSHV infection on neuronal SH-SY5Y cells and analyze differentially expressed genes.

Methods: KSHV was collected from islk.219 cells. TaqMan real-time PCR was used to quantify KSHV copy numbers. KSHV was used to infect SH-SY5Y cells. Infection was confirmed by detection of GFP and viral gene expression. The KSHV copy number in supernatants and mRNA of latency-associated nuclear antigen (LANA), ORF26, K8.1A, replication and transcriptional activator (RTA) of infected SH-SY5Y cells was detected by real-time PCR. The proteins were detected by immunohistochemistry. Effects of KSHV on cell proliferation were detected by MTT and Ki-67 staining. Migration was detected by Transwell assays. The cell cycle was detected by flow cytometry. The expression of CDK4, CDK6, cyclin D1 and p27 were detected by western blots. Cell cycle proteins were detected again in LANA-overexpressing SH-SY5Y cells. Transcriptome sequencing was used to screen differentially expressed genes from KSHV-infected and uninfected SH-SY5Y cells. Components of the Notch signaling pathway were detected by western blots.

Results: SH-SY5Y cells were successfully infected by KSHV and maintained the ability to produce virions. LANA, ORF26, K8.1A and RTA were expressed in KSHV-infected SH-SY5Y cells. After infection, cell proliferation was increased, but migration ability was downregulated and KSHV infection promoted the G0/G1 phase (P<0.05). CDK4, CDK6 and cyclin D1 were upregulated, and P27 was downregulated. After over-expression of LANA, expression of cyclin D1 and CDK4 increased. Transcriptome sequencing showed that 11,258 genes were upregulated and 1,967 were downregulated in KSHV-infected SH-SY5Y cells. The Notch signaling pathway was related to KSHV-infected SH-SY5Y cells, and western blots confirmed expression of Notch1, NICD, RBP-JK and Hes1 increased in KSHV-infected cells.

Conclusion: KSHV can infect SH-SY5Y cells and promote proliferation. KSHV infection upregulated expression of Notch signaling pathway proteins, which may relate to improved cell proliferation.

Background

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesivirus-8 (HHV-8) is a human oncogenic virus of Kaposi’s sarcoma (KS). KSHV is linked to other lymphoproliferative malignancies, including primary effusion lymphoma (PEL) and multicentric Castleman’s disease (MCD). Most reports on KSHV infection mainly focus on endothelial cells and B lymphocytes [1].

Acquired immunodeficiency syndrome (AIDS) patients are susceptible to KSHV [2], and some patients have neurological symptoms such as memory loss, learning diability and behavioral changes [3]. Little attention has been devoted to whether KSHV accesses or causes central nervous system (CNS) symptoms or not. A few reports based on PCR methods suggest the neuroinvasive potential of KSHV.

These studies include detection of KSHV DNA in dorsal root ganglia in KS patients, or in cerebrospinal fluid from HIV-positive patients [4, 5]. However, these reports are insufficient to unequivocally demonstrate the presence of KSHV in the brain parenchyma, because extensive CNS vascularization and the known tropism of KSHV for immune lineages could result in positive detection.

KSHV is a double-stranded DNA virus with a genome length of about 170 kb [6]. The genome contains at least 90 open reading frames (ORFs), including 15 unique genes named K1 to K15; the others are represented by ORFs [7–9].When host cells are infected by KSHV, the virus exhibits two phases: persistent latent infection and a transient lytic infection. Replication and transcription activator (RTA) encoded by ORF50 is the key switch regulator that controls KSHV reactivation [6]. Gene expression during the latency phase mainly includes latency-associated nuclear antigen (LANA) encoded by ORF73, viral cyclin (v-cyclin) encoded by ORF72, viral FLIP (v-FLIP) encoded by ORF71, kaposin encoded by ORF-K12, and 25 mature viral miRNAs [10]. In the lytic state, the KSHV genome is changed to linear DNA that leads to virus production, enabling the spread of virus. The virus expresses most genes in the lytic state, including ORF26, RTA, and K8.1[11].

In previous study, we paired immunohistochemistry and immunofluorescence with PCR methods to localize KSHV-infected cells in the brain parenchymain asymptomatic HIV-positive individuals [12]. We showed that KSHV infects human neurons, suggesting that the CNS system could be a potential reservoir for KSHV. Whether KSHV infection has some effects on the CNS and neuronal cells needs to be investigated further.

To detect the association between KSHV infection and CNS diseases, we used KSHV to infect SH-SY5Y cells. Our study describes that KSHV infected SH-SY5Y cells *in vitro*. We found that KSHV infection promoted the proliferation of SH-SY5Y cells and accelerated the G0/G1 phase by promoting expression of cyclin proteins. We also screened differentially expressed genes by transcriptome sequencing and detected the expression level of Notch 1 signaling pathway components. This study revealed the effects of KSHV infection on neuronal cells and provided a theoretical basis for therapeutic targeting of CNS disease caused by KSHV infection.

Results

SH-SY5Y cells can be infected by KSHV

To infect SH-SY5Y cells *in vitro* using KSHV, KSHV virus was added to the supernatant of normal cultured SH-SY5Y cells. Fluorescence was used to confirm infection. The KSHV in islk.219 cells is a recombinant virus. It has an EF-1α promoter that expresses GFP to reflect the latent state and a PAN promoter that expresses RFP to reflect the lytic state. Infected cells expressed GFP. Some infected cells expressed RFP at same time (Fig. 1A). KSHV-infected SH-SY5Y cells were named SK-RG. The morphology of SK-RG cells changed from aggregate to single-scatter growth. Cells become rounded and synapses were shorter (Fig. 1B). To determine if SK-RG cells had the ability to produce virus, we collected the supernatant of lytic islk.219 and SK-RG cells after confirming infection and culturing 7 days. We extracted KSHV DNA. The

KSHV copy number was (5.8 ± 0.6)×10^6 from the supernatant of induced islk.219 cells and (3.5161 ± 0.2) ×10^6 from the supernatant of SK-RG cells (Fig. 1C).This result indicated that SH-SY5Y cells could be infected by KSHV and infected cells released virus into the supernatant.

KSHV-infected SH-SY5Y cells express viral genes

We used real-time PCR to detect mRNA levels of KSHV lytic genes ORF26, ORF50, and K8.1A and latent gene LANA. SK-RG cells expressed all of the genes. We used islk.219 cells as the positive control (Fig. 2A). Protein levels of K8.1A, RTA and LANA were detected by IHC, with lytic islk.219 cells as the positive control and SH-SY5Y cells as the negative control group. SK-RG and positive controls stained positively for K8.1A, RTA and LANA (Fig. 2B). This result indicated that KSHV-infected SH-SY5Y cells expressed viral genes.

KSHV-infected cells have faster growth kinetics

The same number of SK-RG and SH-SY5Y cells were inoculated, and cell proliferation was detected by an MTT method. After 3 days, absorbance was significantly higher for SK-RG cells than SH-SY5Y cells (P< 0.05) (Fig. 3A). This result meant that KSHV infection promoted cell growth. We used IHC to detect expression of Ki-67 in SK-RG and SH-SY5Y cells. Ki-67 stained strongly in SK-RG cells (Fig. 3B), indicating that KSHV infection promoted the proliferation of SH-SY5Y cells. The cell cycle is closely related to cell proliferation. We detected the cell cycle of SK-RG and SH-SY5Y cells by flow cytometry. The proportion of G0/G1 phase SK-RG cells was lower than the proportion of SH-SY5Y cells (P< 0.05) (Fig. 3C-D), demonstrating KSHV-infected cells accelerated the G0/G1 phase. In transwell arrays, we found the number of SK-RG cells that passed through the membrane was less than the number of SH-SY5Y cells (Fig. 3E). These results showed that KSHV increased cell proliferation through promoting the cell cycle.

KSHV infection increases expression of cyclin proteins

To further analyze the mechanism of cell cycle variation, we used western blots to analyze the expression of CDK4, CDK6, cyclin D1 and p27. Expression of CDK4, CDK6 and cyclin D1 was significantly higher in SK-RG cells than in SH-SY5Y cells (Fig. 4A-B) (P< 0.05), and expression of p27 was lower than in SH-SY5Y cells (P< 0.05).

The mechanism of virus pathogenicity could through alter the expression of the host gene. LANA is an important KSHV gene related to the replication cycle and pathogenicity of the virus. We detected the effects of LANA on cyclins. We overexpressed LANA in SH-SY5Y cells and detected expression of CDK4, CDK6, cyclin D1 and p27. Expression of CDK4 and cyclin D1 were significantly higher in the transfection than the control group, while expression was not significantly different for CDK6 and p27 protein (Fig. 4C-D).

Differentially expressed genes were screened by transcriptome sequencing of SK-RG and SH-SY5Y cells

For analysis of differentially expressed genes in the KSHV-infected SH-SY5Y cells, we used transcriptome sequencing on the cells. The datasets generated for this study can be found in the sequence read archive (SRA). The BioProject accession number is PRJNA627558. The SRA accession number for 3 uninfected groups are SRR11596331, SRR11596330, SRR11596329; for 3 uninfected groups are SRR11596328, SRR11596327, SRR11596326. A total of 13,225 differential genes were detected in the SK-RG group (Fig. 5A). In the SK-RG group, 11,258 genes were upregulated and 1,967 were downregulated (Fig. 5B). GO analysis showed that based on molecular function, upregulated genes were primarily involved in binding, catalytic activity and transcription regulator activity. Based on biological process, the upregulated genes primarily participated in response to cellular process, biological regulation and development process function (Fig. 5C). KEGG pathway analysis found that the Notch 1 signaling pathway was closely related with the KSHV infected SH-SY5Y cells (Fig. 5D).

KSHV infection increased the expression of Notch1 signaling pathway components

According to the KEGG pathways results, the Notch1 signaling pathway showed a strong correlation, so we detected the expression of Notch1 signaling pathway proteins Notch1, NICD, RBP-JK and Hes1 by western blots. Expression of Notch1, NICD, RBP-JK and Hes1 was higher in SK-RG cells than in SH-SY5Y cells (Fig. 6A-B). This result meant that KSHV infection helped to up regulate the expression of Notch1 signaling pathway components.

Discussion

KSHV is an important member of the γ-herpesvirus family. The tropism of KSHV includes endothelial, epithelial and some immune cell types, such as B cells and macrophages[10].

Evidence indicates that human herpesvirus can enter the central nervous system through peripheral axons and blood flow. Herpes simplex virus, human herpesvirus 4 and human herpesvirus 6 are associated with many neurological diseases, including primary central nervous system lymphoma, multiple sclerosis, Alzheimer's disease and cerebellar ataxia. A feature of this virus family is its ability to persist in human host cells indefinitely in a latent state [15–17].

HIV positive patients are very susceptible to KSHV. In addition, 24% of HIV-positive patients have symptoms of aseptic meningitis [3].Since most herpesviruses exhibit some degree of neurotropism, studies considered if KSHV infects the nervous system and causes some dysfunction. Some research also investigated KSHV infection of the nervous system. High detection of KSHV in paravertebral ganglia was reported in seven patients with AIDS who had Kaposi’s sarcoma [18]. N S Brink, *et al.* [4] detected Epstein-Barr virus and KSHV DNA in the cerebrospinal fluid of patients infected with HIV using PCR and found few patients had KSHV infections. The neuroinvasive and neuropersistent potential of KSHV was implied from further findings. Said JW, *et al*. [5] reported three patients with encephalitis, two of whom were positive for HIV and had associated KSHV/HHV-8 sequences using PCR assays. PAUL K.S, *et al*.[19] collected postmortem brain tissues from 30 patients for KSHV detection by PCR and found up to 42 of

300 samples (14.0%) were KSHV positive. However, these studies did not directly prove that KSHV infects neuron cells and causes functional disorder.

To study the effects of KSHV in neuronal cells, we constructed a KSHV-infected cell model using SH-SY5Y cells named SK-RG cells. KSHV to infect SH-SY5Y cells was collected from islk.219 cells. The KSHV in islk.219 cells is a recombinant virus. It has an EF-1α promoter that expresses GFP to reflect the latent state and a PAN promoter that expresses RFP to reflect the lytic state. When KSHV infected, cells express GFP. If the virus is in the lytic state, the infected cells express RFP [20]. In our study, interestingly, after KSHV infection, some infected SH-SY5Y cells steadily expressed both RFP and GFP without induction. That result meant that KSHV can infected SH-SY5Y cells and spontaneously entered into the lytic state.

To further study these findings, we continuously detected expression of KSHV genes. Latent KSHV expresses only a limited number of genes to evade the host cell's immune response. The lytic virus major expresses different genes than the latent state [21]. We detected mRNA from the latent gene LANA and the lytic genes ORF26, RTA, and K8.1A using real time-PCR. SK-RG cells expressed mRNA from both the latent and the lytic genes. We further detected the protein level of LANA, RTA, ORF26 and K8.1A using IHC. The proteins were also expressed in SK-RG. All these results showed that KSHV successfully infected SH-SY5Y cells and some cells expressed both latent and lytic genes.

In the process of culturing SK-RG cells, we found that the cells grew faster than noninfected cells. Therefore, we detected cell proliferation by MTT and Ki-67 staining assays. The results showed that proliferation of KSHV-infected cells was faster than in uninfected cells. Manipulation of the cell cycle is a commonly employed strategy of viruses to achieve a favorable cellular environment during infection [22]. To investigate the reason for cell proliferation, we used flow cytometry to detect the cell cycle. The proportion in G0/G1 phase was significantly lower for in SK-RG cells than SH-SY5Y cells. G0/G1 phase is the first gap for cells to organize prior to DNA replication. Any decisive events during G0/G1 phase determine if the cell proceeds to division, pauses, or exits the cell cycle [23]. The cell cycle is also critical for tumorigenesis and progression. Its disorders reduce the stability of the genome and lead to abnormal proliferation of tumor cells [24, 25].

Cyclin proteins are closely related with the cell cycle. Cyclins have the ability to activate cyclin dependent kinases (CDKs) [26]. Cyclin D1 is frequently deregulated in cancer and is a biomarker of cancer phenotype and disease progression. To analyze the effect of KSHV infection on the cell cycle, we examined expression levels of cyclin D1, CDK4, CDK6 and p27 by western blots. P27 is an inhibitor whose specific late G1 destruction allows progression of the cell across the G1/S boundary [27]. Expression levels of cyclin D1, CDK4 and CDK6 in SK-RG cell were significantly higher, and expression of p27 was lower than in the SH-SY5Y group. This result indicated that KSHV infection promoted expression of cyclin D1, CDK4, and CDK6 and decreased expression of p27.This finding may be the reason that KSHV infection altered the cell cycle.

LANA is a major latent gene and transcription factor of KSHV [28]. Wei, F, et al.[22] reported that LANA is an oncoprotein that steers KS and PEL cell cycle-related events including proliferation and apoptosis by

interacting with cellular and viral factors. LANA is also reported to promote the proliferation of MCD [29] and endothelial cells [30].We used a LANA plasmid for overexpression in SH-SY5Y cells and detected expression of CDK4, CDK6, cyclin D1 and p27. We found that LANA promoted expression of cyclin D1 and CDK4. This promotion may explain why KHSV promoted the cell cycle and increased proliferation.

The pathogenic mechanism of viruses always involves in alteration of the expression of host genes. We screened for genes differentially expressed in SK-RG and SH-SY5Y cells using transcriptome sequencing. We found 11,258 upregulated and 1967 downregulated genes in SK-RG cells compared with SH-SY5Y cells. According to KEGG pathway analysis, the Notch1 signaling pathway was activated in SK-RG cells and releted with KSHV infection. The Notch signaling pathway is important in embryonic development, and in adult tissues, regulates cell proliferation, differentiation and apoptosis [31, 32]. Yingying Hou [33] reported that overexpression of Notch1 promotes cell growth in nasopharyngeal carcinoma cells. Du X [34] reported that Notch1 is overexpressed in gastric cancer tissues and related to cancer progression. The Notch signaling pathway is also critical for the development of KS. Curry CL [35] reported that expression of Notch 1 is elevated in KS tumor cells. KSHV is reported to usurp the Notch signaling pathway to inhibit other viruses in neighboring cells [36]. Zhiming Zhuang [37] reported that Notch 1 acts as an activator for cell proliferation and a suppressor for cell apoptosis through the Akt/mTOR signaling-dependent pathway in renal cell carcinoma. Notch1 also mediates the chemoresistance response and strengthens proliferation capacity in lung adenocarcinoma cells through negative regulation of miR-451 by the transcription factor AP-1 [38]. Based on these reports, we compared the expression level of Notch1 and pathway components NICD, RBP-JK and Hes1 in SK-RG and SH-SY5Y cells. We found that expression of Notch1, NICD, Hes1 and RBP-JK was significantly higher in SK-RG cells, implying that the mechanism of KSHV pathogenicity is involved with the Notch1 pathway.

We showed that KSHV can successfully infect SH-SY5Y cell *in vitro*. More importantly, we found that KSHV infection increases proliferation and promotes the cell cycle of SH-SY5Y cells. The expression of CDK4, CDK6, cyclin D1, and components of the Notch signaling pathway was upregulated, which could be helpful for studying the effects of KSHV on neuronal cell function.

In the future, we will study if the increased growth of neuronal cells caused by KSHV infection influence cell function such as electrophysiological characteristics. We will analyze the regulation mechanisms of the Notch signaling pathway by KSHV infection in neuronal cells.

Conclusion

KSHV can successfully infect SH-SY5Y cells in vitro, and can promote cell proliferation by up-regulating the Notch signaling pathway.

Methods

Cell cultures

The SH-SY5Y cell line was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The SH-SY5Y cell line was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. The KSHV positive cells, iSLK.219, were kindly gifted from Prof. Ke Lan. islk.219 cells and KSHV-infected SH-SY5Y cells were cultured in the presence of 6μg/ml puromycin (Gibco, Carlsbad, CA, USA), 100ug/ml G418 (Solarbio, Beijing, China) and 100μg/ml hygromycin (Invitrogen, Carlsbad, CA, USA). All cells were incubated at 37°C with 5% CO_2.

KSHV induction and virion extraction

To induce KSHV lytic replication, islk.219 cells were treated with 1μg/ml doxycycline (Sigma Aldrich, St. Louis,MO, USA) and 1.25 mM sodium butyrate (Sigma Aldrich, St. Louis,MO, USA) for 72h. Virus-containing supernatant was collected and residual cells removed by centrifugation (3000rpm, 15 min) before filtering with a 0.45-μm filter. Add the same volume of cold PEG-it Virus Precipitation Solution (System Biosciences, Shanghai, China) at 4ºC overnight. Centrifuge supernatant/PEG-it mixture at 1500×g for 30 minutes at 4ºC. Aspirate supernatant and then resuspend the virus pellet in sterile PBS. Virus stocks were stored at -80ºC.

Supernatant KSHV DNA extraction

Supernatant was collected from KSHV-infected cells. Supernatant was treated with deoxyribonuclease I (DNaseI) (Invitrogen, ThermoFisher Scientific,Warrington, UK) at 37℃ to remove residual cellular DNA. KSHV DNA was isolated from the supernatant using a phenol/chloroform extraction protocol. Add the same volume of phenol: chloroform: isoamyl alcohol (25:24:1) to the virion production, centrifuge at room temperature for 5 minutes at 16,000g, add 1μL glycogen (20μg/μL),7.5mol/L NH_4OAc (0.5×volume of sample) and 100% ethanol (2.5×volume of sample + NH_4OAc), store at -20°C overnight to precipitate the DNA. Centrifuge at 4°C for 30 minutes at 16,000g to pellet the DNA, remove the supernatant and add 150μL 70% ethanol. Centrifuge at 4°C for 2 minutes at 16,000 g and then remove the supernatant. Dry the DNA pellet at room temperature for 10 minutes. Resuspend the DNA pellet in 100μL of TEN buffer and centrifuge briefly to collect the KSHV DNA.

Taqman real-time PCR

TaqMan real-time PCR assays were performed in final volume 20 μL using TaqMan real-time PCR kits (Takara biomedical technology, Beijing, China), with 6.2 μL RNA-free water, 10 μL Premix Ex Taq (Probe qPCR) (2X), each primer and TaqMan probe at 10 μM, 0.2 μL ROX Reference Dye II and 2 ng DNA template for all samples. Amplification was with primers and probe sequences in Table 1. TaqMan real-time PCR reactions were performed on an Applied Biosystems 7500/7500 Fast Real-Time PCR (Applied Biosystems, Carlsbad, CA, USA). Sensitivity was determined by testing decreasing DNA quantities of ORF 26 plasmids (10-fold dilutions from 10^2 to 10^8 ng/μL). PCR was performed using cycling conditions: 50ºC for 2 min, 95 ºC for 10min; 40 cycles of 95ºC for 15s, 60ºC for 1min.

Table 1
List of oligonucleotide primers and probes

Target Gene	ID	Sequence (5'-3')
Primer		
K8.1A for real-time PCR	Forward	AAAGCGTCCAGGCCACCACAGA
	Reverse	GGCAGAAAATGGCACACGGTTAC
RTA for real-time PCR	Forward	GAGTCCGGCACACTGTACC
	Reverse	AAACTGCCTGGGAAGTTAACG
ORF26 for real-time PCR	Forward	CGAATCCAACGGATTTGACCTC
	Reverse	CCCATAAATGACACATTGGTGGTA
LANA for real-time PCR	Forward	AGCCACCGGTAAAGTAGGAC
	Reverse	AGCCACCGGTAAAGTAGGAC
β-actin for real-time PCR	Forward	CGGAACCGCTCATTGCC
	Reverse	ACCCACATCGTGCCCATCTA
ORF 26 for Taq-man real-time PCR	Forward	CGAATCCAACGGATTTGACCTC
	Reverse	CCCATAAATGACACATTGGTGGTA
Probe		
ORF 26 for Taq-man real-time PCR		5'FAM/CCCATGGTCGTGCCGCAGCA/3'BHQ-1

Infection of SH-SY5Y cells by KSHV

KSHV virus from islk.219 cells was used to infect SH-SY5Y cells at a density of 1×10^6 KSHV virus copy number to 1×10^3 SH-SY5Y cells. Infected SH-SY5Y cells were cultured for 3 days. Observation of green fluorescent protein (GFP) and red fluorescent protein (RFP) was performed using a fluorescence microscope and infected SH-SY5Y cells were named SK-RG.

RNA extraction and real-time PCR

Total RNA from cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. RNA integrity was detected using 1% agarose gel electrophoresis. Total RNA was reverse transcripted to cDNA using a Revert Aid First Strand cDNA Synthesis kit (ThermoFisher, Carlsbad, CA, USA) following the manufacturer's protocol and every sample had 1.0 μg total RNA as template. The mRNA level of K8.1A, RTA, ORF26 and LANA was quantified using a SYBR Green PCR kit (QIAGEN, Hilden, GER) following the manufacturer's protocol and primers in Table 1. The amplification protocol included an initial heat activation step at 95℃ for 5 min, followed by 35 cycles of 95℃ for 30s

and combined annealing/extension at 60°Cfor 30s. Results were calculated as $2^{(-\triangle CT)}$ values and PCR reaction specificity was confirmed by melting curve analysis.

Immunocytochemistry

Cells (2×10^6) were centrifuged at 800rpm for 5 min. Aspirated the supernatant and fixed with 4% paraformaldehyde for 20min, centrifuge at 800rpm for 5 min and wash cells with 1×PBS, then permeabilized with 0.1% Triton-X-100 for 15min. Removed the supernatant and resuspended the cells in 1× PBS. Dropped the cell solution in 12 wells slides, 10 μl per well. Slides were blocked overnight at 4°C with the indicated antibodies were diluted in Bond Primary Antibody Diluent (Leica, Shanghai, China): anti-LANA (1:500dilution, MBL, Japan), anti-RTA (1:400dilution, ABBIOTEC, San Diego, CA,USA), anti-K8.1A (1:400dilution, Santa, USA). Slides were washed with 1×PBS, and incubated with horseradish peroxidase-labeled polymeric secondary antibody. Dako REALTM EnVisionTM Detection System (Dako, Glostrup, DK) was used for chromogen deposition and hematoxylin was used for counterstaining.

Mtt Assays

Cells (2000) were incubated in wells of a 96-well plate. At days 1, 2, 3, 4, and 5, 20μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Solarbio, Beijing, China) was added to each well. After 4h at 37°C in the dark, MTT medium was removed and dimethyl sulfoxide (Solarbio, Beijing, China) 100μl/well was added. Absorbance of dyed solutions was measured at optical density 490nm (OD_{490}).

Transwell assays

A total of 1×10^4 SK-RG and SH-SY5Y cells were inoculated into 24-well plates with 8-μm pore size chamber inserts. Cells were seeded in 600 μL serum-free DMEM into the upper chamber of each well and 20% FBS was added to the lower chamber before incubation at 37°C in 5% CO_2 for 48h. At the end of the incubation, cells were fixed in methanol for 20 min and stained in 0.1% crystal violet for 30 min for microscopic inspection.

Flow cytometry

Digested the SK-RG and SH-SY5Y cells with trypsin without EDTA, centrifuged at 800rpm for 5 min. Fixed the cells in ice-cold methanol store at -20°C overnight. Centrifuged the cells at 800rpm for 5 min, resuspended the cells with 500μl 1× PBS, add 5μl PI and 5μl RNAseA, incubate at 37°C in the dark for 30min, the cell cycle were detected by flow cytometry (BD Biosciences, New York, USA).

Transcriptome Sequencing

Transcriptome sequencing was performed by BGI Shenzhen Company using three groups of SK-RG cells and three groups of SH-SY5Y cells. A total of six samples were measured using the BGISEQ-500 platform and filtered using SOAP nuke from BGI Company. After obtaining clean reads, we used hierarchical indexing for spliced alignment of transcripts to compare the clean reads to the reference genome sequence and used the integrative genomics viewer genome to browse to look at the alignment of reads with the genome.

BGI tested DEG according to the method described in Wang L, et al[13]. The DEGseq method is based on a Poisson distribution. DEGseq was used to normalize the data and screen for differentially expressed genes. The *P*-value was required to be less than 0.05 for removing low-repeat differentially expressed genes. Fold-changes were defined as > 2 for upregulation and < 0.5 for downregulation. Horizontal clustering and gene ontology (GO) were used to calculate the quantity of genes in different nodes. Pathway enrichment analysis of DES target genes used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Western blots

Western blotting used polyvinylidenedifluoride membranes (PVDF) and 5% nonfat milk as blocking reagent, as described previously [14]. Primary antibodies were: anti-CDK4 (1:1000 dilution, Bioward Technology, Nanjing, China), anti-CDK6 (1:500 dilution, Bioward Technology, Nanjing, China), anti-cyclin D1(1:500 dilution, Bioward Technology, Nanjing, China), anti-p27(1:1000 dilution, Bioward Technology, Nanjing, China), anti-Notch1(1:1000 dilution, Cell Signaling Technology, USA), anti-NICD(1:1000 dilution, Abcam, USA), anti-RBP-JK (1:1000 dilution, Abcam, USA), anti-Hes1 (1:1000 dilution, Cell Signaling Technology, USA), and anti-β-actin (1:1000 dilution, ZSGB-BIO, Beijing, China); and secondary antibody was goat anti-mouse IgG (1:10,000 dilution, ZSGB-BIO, Beijing, China) or goat anti-rabbit IgG (1:10000 dilution, ZSGB-BIO, Beijing, China).

Transfection assays

LANA plasmid was from Hangzhou Normal University. SH-SY5Y cells were transfected with 2 μg LANA or vector using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24, 48 and 72h, cells were lysed and RNA was extracted for real-time PCR to determine transfection efficiency (Supplement Fig. 1).

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Independent *t*-tests were used for data measurement. Error bars represented standard deviations. $P < 0.05$ was considered statistically significant.

Abbreviations

Abbreviations	Full acronym
KSHV	Kaposi's sarcoma-associated herpesvirus
GFP	green fluorescent protein
LANA	latency-associated nuclear antigen
ORF	open reading frame
RTA	Replication and transcription activator
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
CDKs	cyclin dependent kinases
NICD	Notch Intracellular Domain
RBP-Jκ	recombination signal sequence-binding protein Jkappa
HHV-8	human herpesivirus-8
KS	Kaposi's sarcoma
PEL	primary effusion lymphoma
MCD	multicentric Castleman’s disease
AIDS	Acquired immunodeficiency syndrome
CNS	central nervous system
v-cyclin	viral cyclin
v-FLIP	viral FLIP
DMEM	Dulbecco’s modified Eagle medium
FBS	fetal bovine serum
DNaseI	deoxyribonuclease I
RFP	red fluorescent protein
GO	gene ontology
PVDF	polyvinylidenedifluoride membranes
KEGG	Kyoto Encyclopedia of Genes and Genomes

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors contributions:

Dongdong Cao, Shuyuan Wu and Xiaolu Wang carried out the experiments, analyzed the data and drafted the manuscript. Dongmei Li designed the experiments and critically revised the manuscript for important intellectual input. Zemin Pan, Lei Yang and Xiaohua Tan were accountable for all aspects of the work in ensuring that questions related to the accuracy. Ying Li , Huiling Xu and Zhaofu Wu provided technical support. All authors participated in writing the paper and had final approval of the submitted and published versions.

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Figures

Figure 1

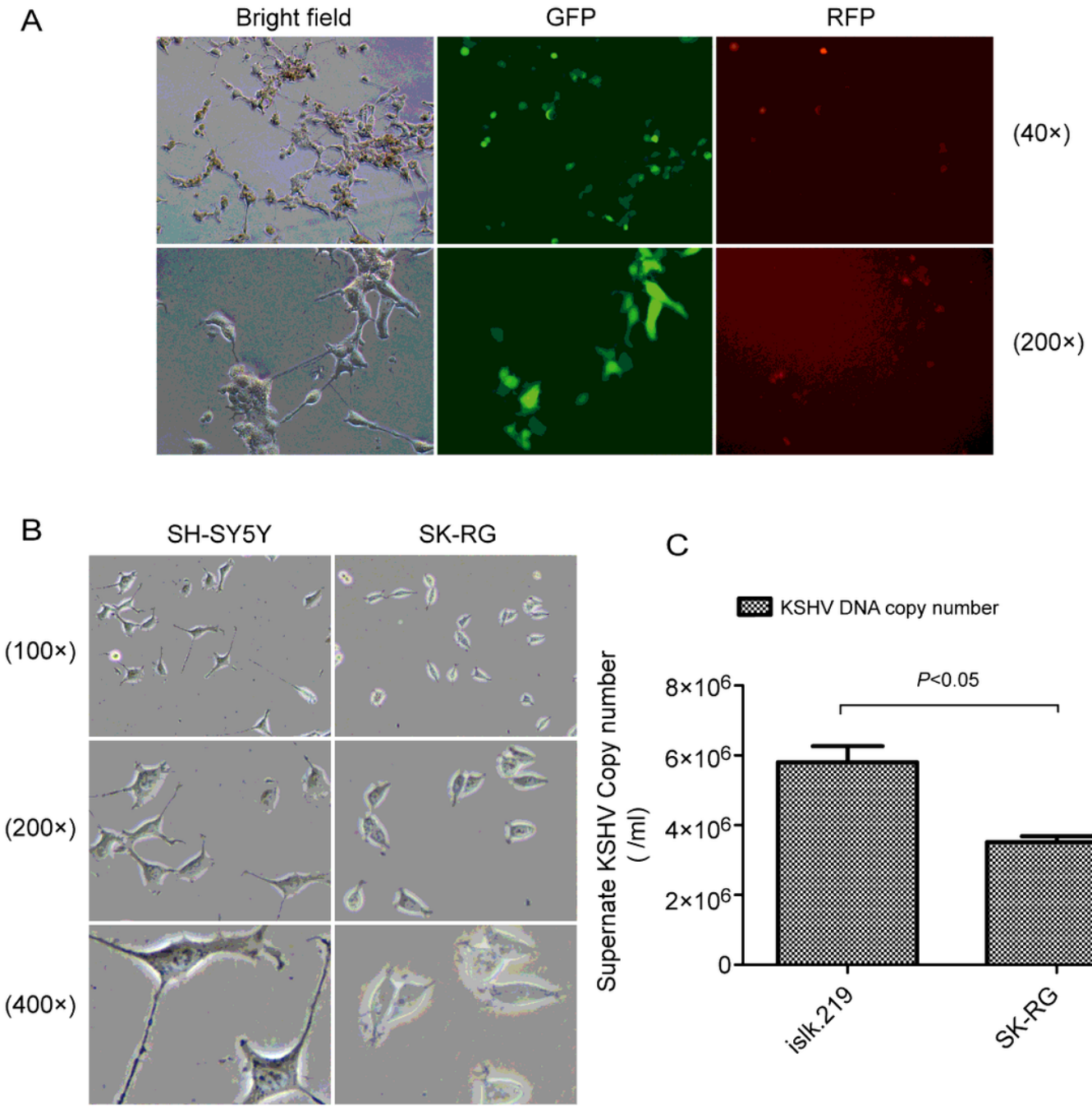

Figure 1

KSHV successfully infects SH-SY5Y cells. (A) SH-SY5Y cells were infected with KSHV for 72 h and named SK-RG cells. GFP fluorescence was used to confirm infection (40×, 200×). Some cells expressed RFP spontaneously. (B) SK-RG and SH-SY5Y cells were cultured and cell morphology compared by microscope. (C) Cell supernatant was collected from lytic induced islk.219 cells and SK-RG cells after confirming infection and culturing for 7 days. KSHV DNA copy number was detected by TaqMan real-time PCR.

Figure 2

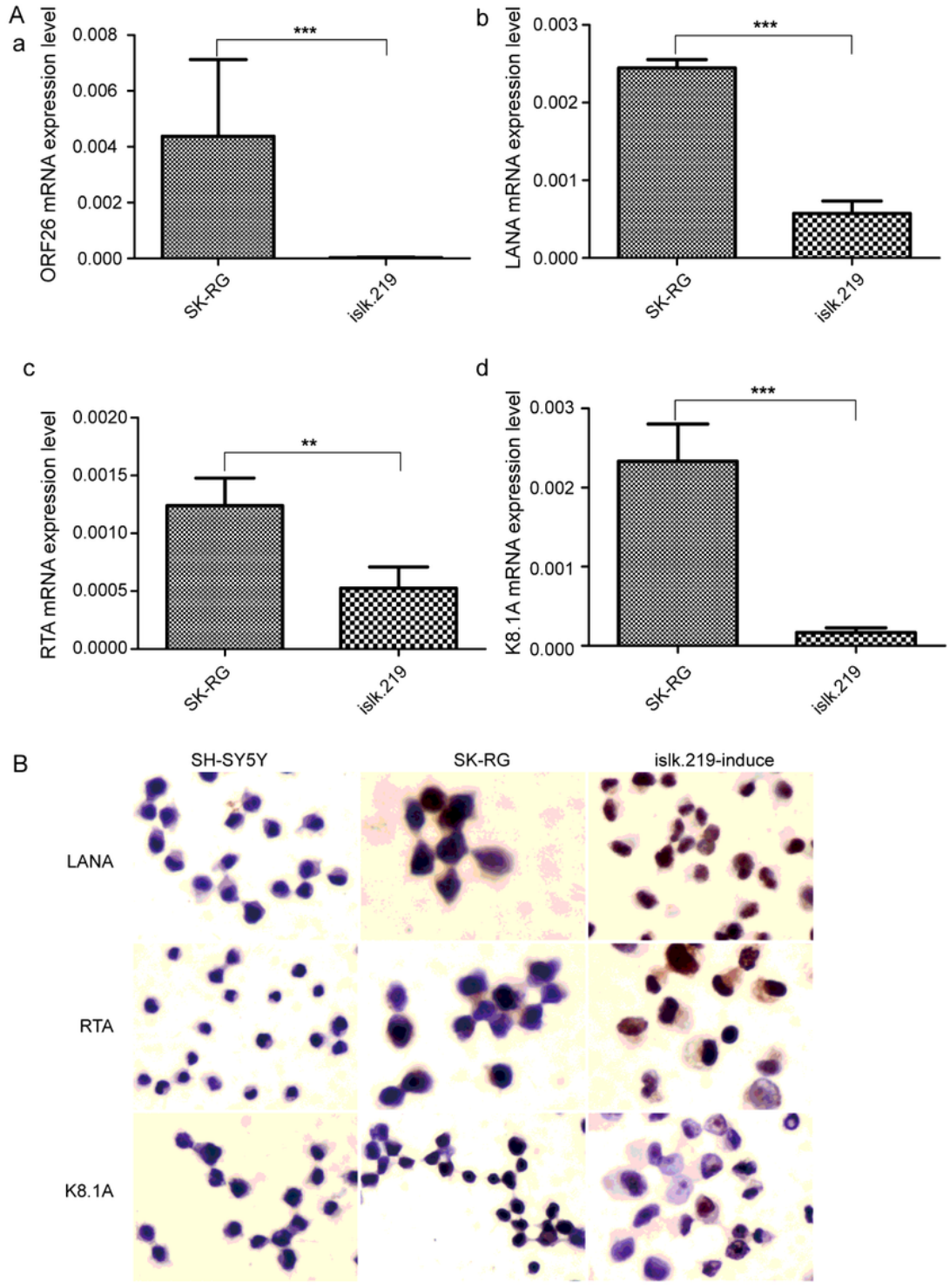

Figure 2

SK-RG cells express viral genes. (A) SK-RG cells express mRNA of the lytic genes ORF26, RTA, and K8.1A and latent gene LANA, as detected by real-time PCR. (B) SK-RG cells express proteins RTA, K8.1A and LANA, by IHC. Lytic-induced islk.219 was the positive control, SH-SY5Y cells were the negative control. * means P<0.05, ** means P<0.01, *** means P<0.001.

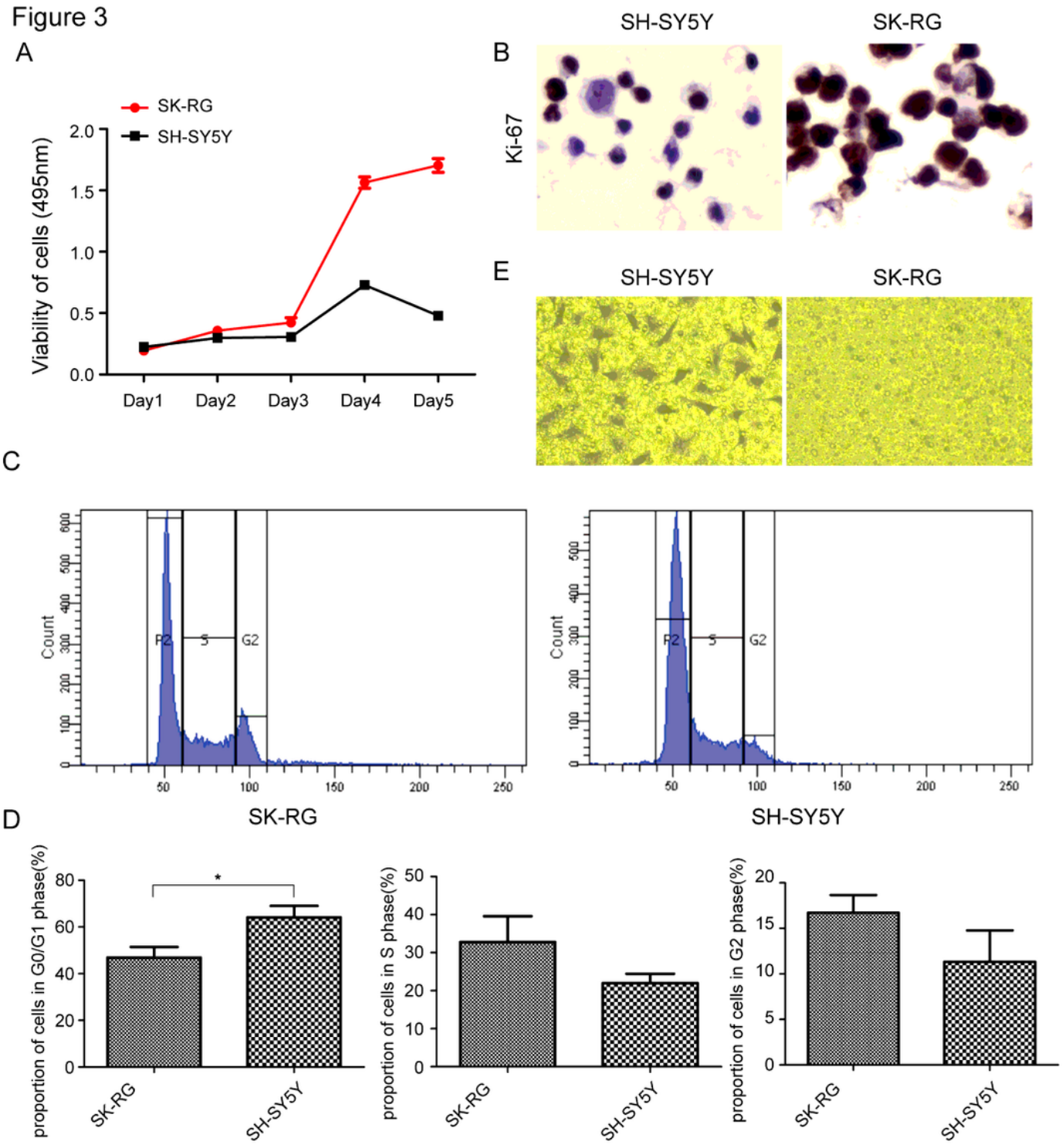

Figure 3

KSHV-infected SH-SY5Y cells have faster growth kinetics. (A) Comparing the proliferationof SK-RG and SH-SY5Y cells by MTT assays showed the growth of SK-RG was faster than SH-SY5Y cells ($P<0.05$). (B) Comparing Ki-67 expression in SK-RG and SH-SY5Y cells by IHC showed staining of Ki-67 was stronger in SK-RG cells. (C-D) Flow cytometry to detect cell cycle showed that KSHV infection promoted G0/G1

progression (P<0.05). (E) Transwell assays to detect migration of SK-RG and SH-SY5Y cells showed migration of SK-RG was decreased.

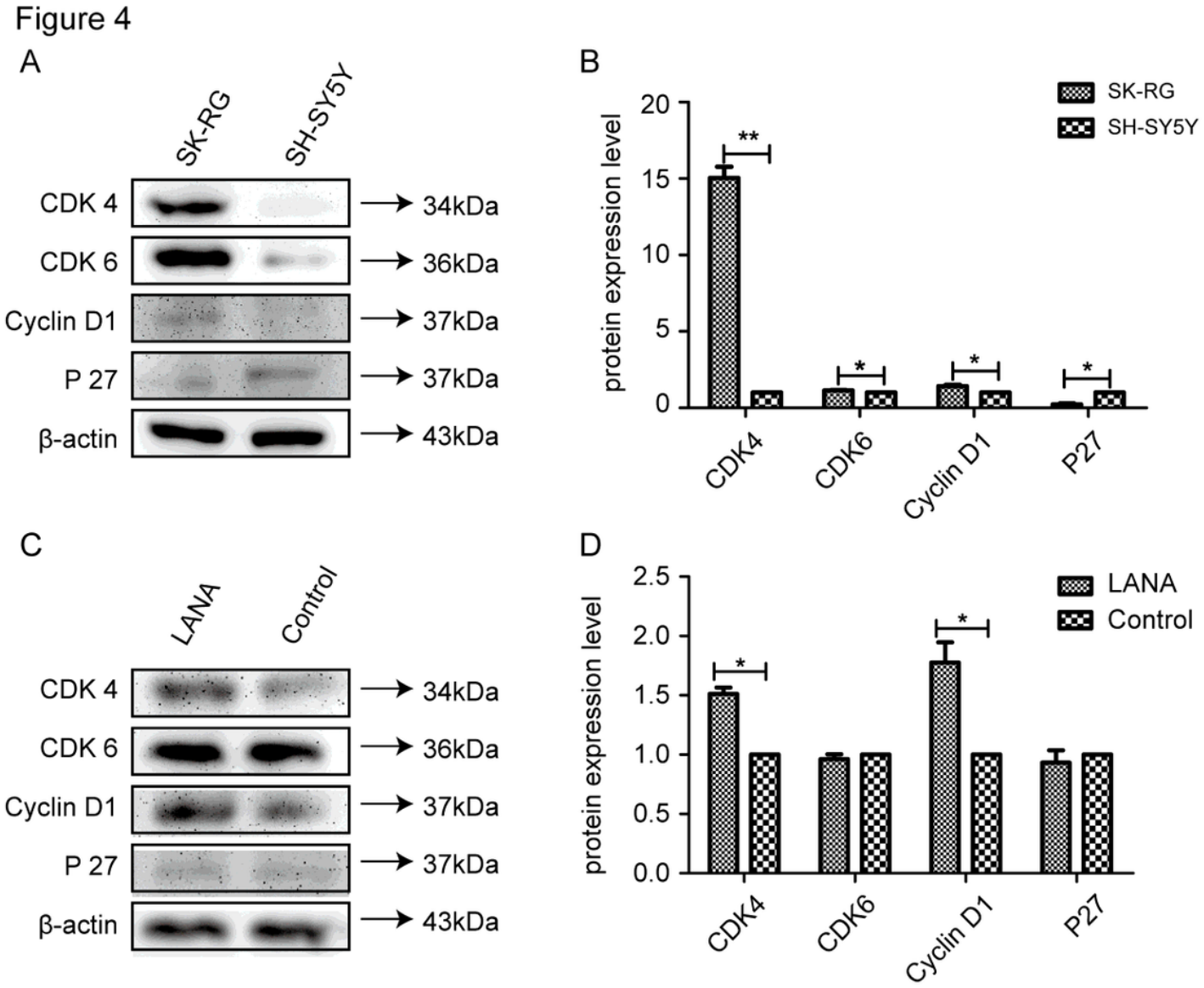

Figure 4

KSHV infection promoted overexpression of cyclin proteins. (A-B) Western blots to analyze expression of CDK4, CDK6, cyclin D1and p27 in SK-RG and SH-SY5Y cells showed expression of CDK4, CDK6 and cyclin D1 was significantly higher and p27 was lower in SK-RG cells than in SH-SY5Y cells (P<0.05). (C-D) Western blots to analyze expression of CDK4, CDK6, cyclin D1 and P27 after LANA overexpression found LANA promoted expression of CDK4 and cyclin D1 (P<0.05).

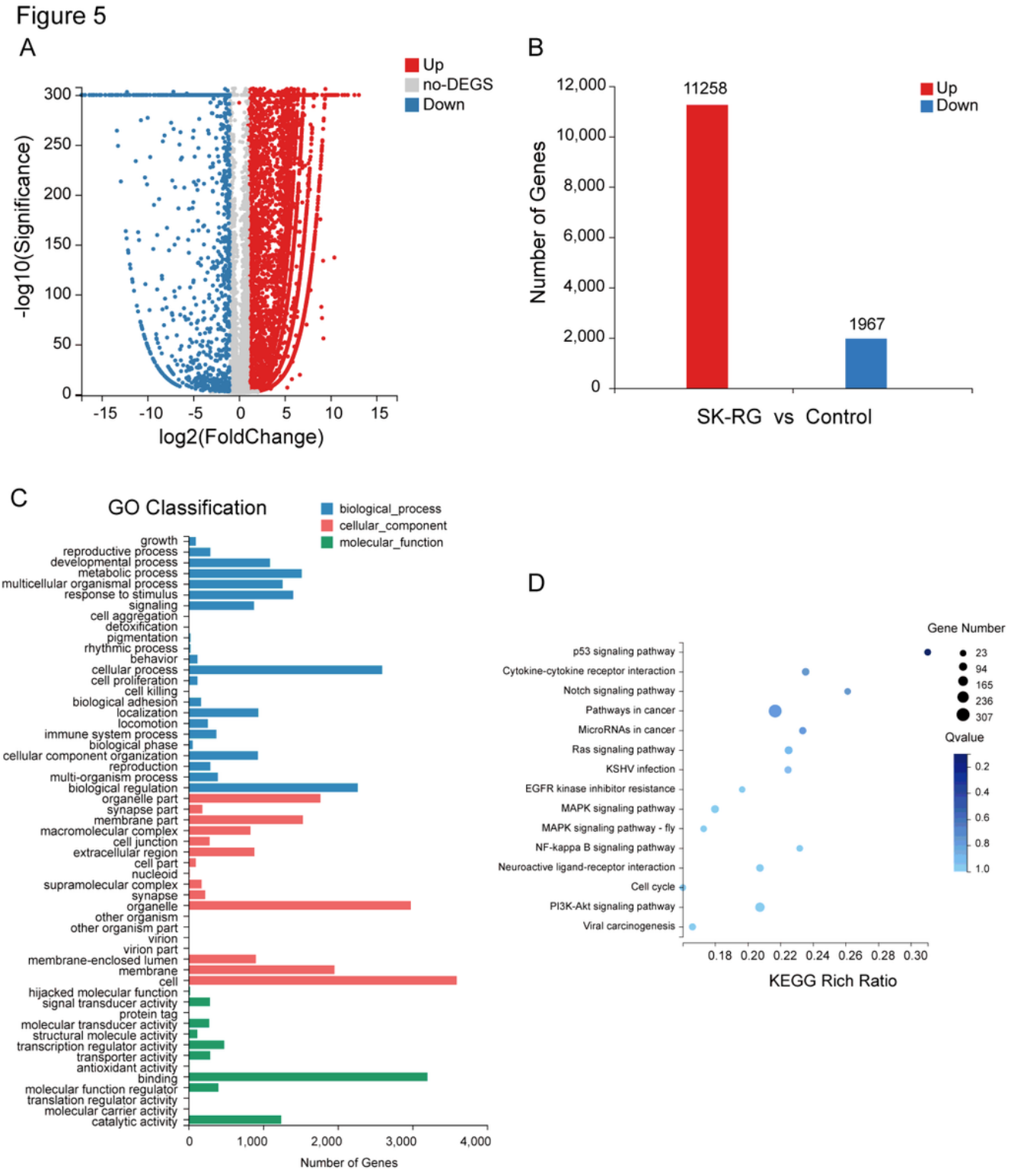

Figure 5

Transcriptome sequencing to screen differentially expressed genes in SK-RG and SH-SY5Y cells. (A) Differentially expressed genes in SK-RG and SH-SY5Y cells by volcano map. Red spots indicate upregulated genes and blues pots indicate downregulated genes in SK-RG cells. (B) Compared with SH-SY5Y, a total of 13,225 differentially expressed genes were detected in the SK-RG group, of which 11,258 were upregulated and 1,967 were downregulated. (C) GO analysis classified differentially expressed

genes as associated with molecular function, cellular components and biological process. (D) KEGG pathway enrichment analysis.

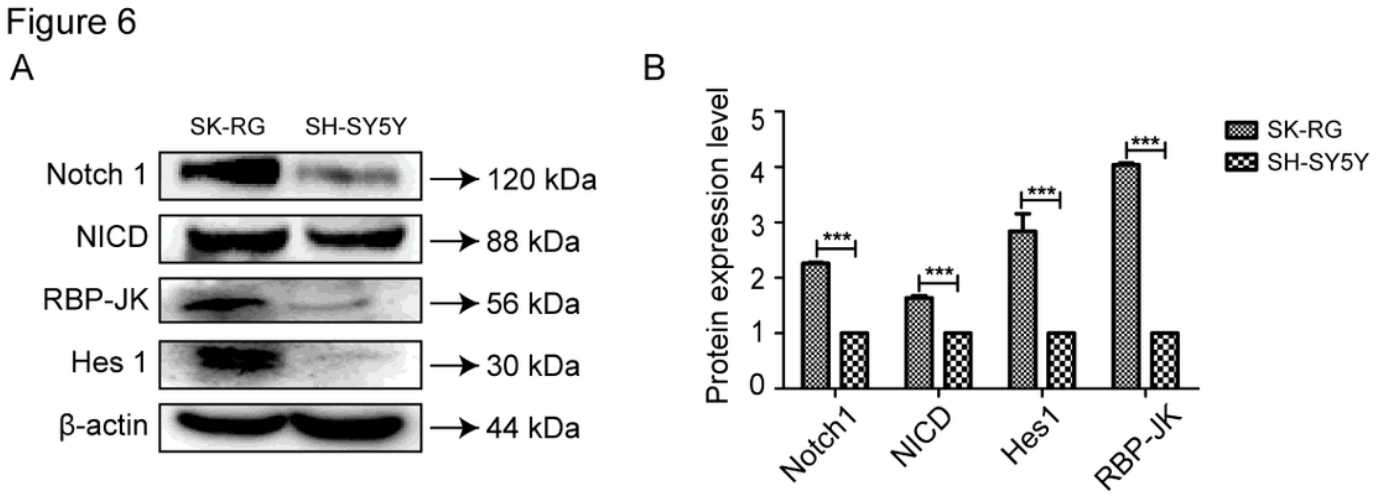

Figure 6

KSHV infection increased component expression of the Notch 1 signaling pathway.(A-B)Western blots to analyze expression of Notch1, NICD, RBP-JK and Hes1 in SK-RG and SH-SY5Y cells showed expression of Notch 1, NICD, RBP-JK and Hes1 was higher in SK-RG than SH-SY5Y cells ($P<0.01$).

Supplementary Files

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- SupplementFig1.tif