ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2005, **37**(4): 215–220 **CN** 31-1940/Q

Induction of Epstein-Barr Virus Lytic Replication by Recombinant Adenoviruses Expressing the *Zebra* Gene with EBV Specific Promoters

Lu CHEN, Juan YIN, Yi CHEN, and Jiang ZHONG*

Department of Microbiology and Microbial Engineering, School of Life Sciences, Fudan University, Shanghai 200433, China

Downloaded from https://academic.oup.com/abbs/article/37/4/215/126 by guest on 24 April 2024

Abstract The latent Epstein-Barr virus (EBV) is found in the cells of many tumors. For example, EBV is detectable in almost all cases, and in almost all tumor cells, of non-keratinizing nasopharyngeal carcinoma. Activating the latent virus, which will result in its lytic replication and the death of tumor cells, is a potential approach for the treatment of EBV-associated cancers. In this study, three recombinant adenoviruses were constructed to express the *Zebra* gene, an EBV gene responsible for switching from the latent state to lytic replication. EBV-specific promoters were used in order to limit *Zebra* expression in EBV-positive cells, and reduce the potential side effects. The EBV promoters used were *Cp*, *Zp* and a dual promoter combining both promoters, *CpZp*. The Zebra protein was detected in HEK293 cells as well as the EBV-positive D98-HR1 cells infected with recombinant viruses. An EBV lytic replication early antigen, EA-D, was also detected in infected D98-HR1, implying the initiation of lytic replication. In the cell viability assay, *Zebra*-expressing adenoviruses had little effect on EBV-negative HeLa cells, while significantly reducing the cell viability and proliferation of D98-HR1 cells. The results indicate that EBV virus promoters can be used in adenovirus vectors to express the *Zebra* gene and induce EBV lytic replication in D98-HR1 cells.

Key words Epstein-Barr virus; recombinant adenovirus; lytic replication; cancer

The Epstein-Barr virus (EBV), or human herpesvirus-4, is a common human virus infecting a large proportion of the population. Numerous researches have associated EBV with various types of human cancer [1]. In southern China and Southeast Asia, EBV is found to be closely associated with nasopharyngeal carcinoma (NPC), in which the latent EBV is present in almost all cases [2,3]. In the other areas of the world, EBV has been linked with Burkitt's lymphoma, Hodgkin's disease, lymphoproliferative diseases in immunodeficient patients, etc. [1,2]. EBV-latent infection has also been found in about 10%–15% of gastric cancer cases [4]. The role of EBV in tumorigenesis is not well understood yet [5,6], although some viral gene products, such as latent membrane protein 1 (LMP1), LMP2, EB nuclear antigen 1 (EBNA1), are suspected of promoting cell transformation [7].

Received: December 22, 2004 Accepted: February 18, 2005

This work was supported by a grant from the "Shuguang" Program of the Shanghai Municipal Education Foundation

*Corresponding author: Tel, 86-21-55664332; Fax, 86-21-65650149; E-mail, jzhong@fudan.edu.cn

The fact that EBV is broadly presented in cancers but rarely found in healthy cells offers an opportunity for targeted cancer therapy [8]. One of the approaches is to activate the lytic replication cycle of the latent EBV, when viral proteins will be expressed at a high level, and progeny viruses will be produced. Lytic EBV replication will damage the cancer cells, and trigger host immune reactions against EBV and the infected cells [9]. Some factors may induce the switch from the latent state to lytic replication, including viral genes such as *Zebra* (*BZLF1*, or *Zta*) and *Rta* (*BRLF1*) [10–12]. Feng *et al.* [13] have used the *CMV* promoter to express *Zebra* and *Rta* genes with adenoviruses, and have observed the specific cell-killing of EBV-positive cells *in vitro* and in nude mice models after adenovirus infection. Since the *CMV* promoter is a strong constitutive promoter active in different cell types, and *Zebra* and *Rta* as transactivators may have unexpected effects on cells, it would be advantageous to use promoters that are only active in EBV-

DOI: 10.1111/j.1745-7270.2005.00032.x

©Institute of Biochemistry and Cell Biology, SIBS, CAS

positive cells for their expression.

In this study, recombinant adenoviruses expressing the *Zebra* gene with EBV promoters were constructed and their potential for inducing EBV lytic replication *in vitro* was evaluated.

Materials and Methods

Amplification and cloning of the EBV *Zebra* gene and promoters

The human lymphoma cell line B95-8 (Shanghai Cell Bank, Committee on Type Culture Collection, Chinese Academy of Sciences, Shanghai, China), a lymphoma cell line with latent EBV infection, was cultured in the presence of 3 mM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 20 ng/ml *n*-butyrate to induce the replication of the EB viral genome. The total cellular DNA was isolated with the Blood/cell genome extraction kit (Shenergy Biocolor Company, Shanghai, China) 3 days after induction, and was used as the template in the polymerase chain reaction (PCR). The PCR primers were designed according to the published sequence (GenBank accession No. NC_001345). The primer pair Z1 (5′-TGGCGGTAAACAATG-3′)/Z2 (5′-GGACCCTGATGAAGAA-3′) was used to amplify the *Zebra* gene coding region containing its native promoter *Zp* (*ZpZebra*), Z3 (5′-CTCGAGTTAGTAAACGAGGCG-3′)/Z4 (5′-AGATCTTGCTATCTTTGCTGAA-3′) was used to amplify the *Zebra* gene coding region only (*Zebra)*, and C1 (5′-TTCTTGATTGCCTCTT-3′)/C2 (5′-GATGAT-TTGTTGGTTA-3′) was used to amplify the virus *Cp* promoter. The *Cp* promoter of EBV is a major promoter for latent gene transcription. The expected PCR products of 1.6 kb, 1.0 kb and 1.2 kb in length were observed, cloned into pUCm-T (Sangon Biotechnology Company, Shanghai, China), and confirmed by DNA sequencing, which was done by Sangon.

Construction of recombinant adenoviruses

Three recombinant adenoviruses were constructed according to the methods described by He *et al.* [14]. First, three shuttle vectors were constructed as shown in **Fig. 1**. Briefly, the 1.6 kb (*ZpZebra*) and 1.2 kb (*Cp*) PCR products were first doubly digested with *Bgl*II/*Nde*I and *Not*I/*Bgl*II, respectively. The plasmid pAdTrackCMV [14], kindly provided by Prof. Y. M. WEN, Shanghai Medical College, Fudan University, Shanghai, China, was first digested with *Not*I, and then partially digested with *Nde*I. The largest fragment was recovered from agarose gel and ligated with digested *ZpZebra* and *Cp* fragments in a three-way ligation reaction to construct the dual promoter shuttle vector pSCpZpZebra. The plasmid pSCpZpZebra was then doubly digested with *Xho*I and *Bgl*II. The largest fragment was recovered and used for ligation with the 1.0 kb (*Zebra*) PCR product that had been digested with the same two restriction enzymes (*Xho*I/*Bgl*II) to produce the shuttle vector with only the *Cp* promoter, pSCpZebra. The plasmid pSCpZpZebra was also doubly digested with *Kpn*I and *Bgl*II, filled in with Klenow DNA polymerase, and self-ligated to produce the shuttle vector with only the *Zp* promoter, pSZpZebra.

The three shuttle vectors, pSCpZpZebra, pSCpZebra and pSZpZebra, were linearized with *Pme*I before being used to transform the *E. coli RecA*$^+$ strain BJ5183/pAdEasy-1, which harbors the plasmid containing the adenovirus genome backbone, pAdEasy-1 [14]. Three plasmids, pAdvCpZpZebra, pAdvCpZebra and pAdvZpZebra, containing the recombinant adenovirus genome derived from the homologous recombination between the shuttle vectors and pAdEasy-1 were obtained from kanamycin- and streptomycin-resistant *E. coli* BJ5183 transformants. These plasmids were digested with *Pac*I to release the recombinant adenovirus genome, which

Downloaded from https://academic.oup.com/abbs/article/37/4/215/126 by guest on 24 April 2024

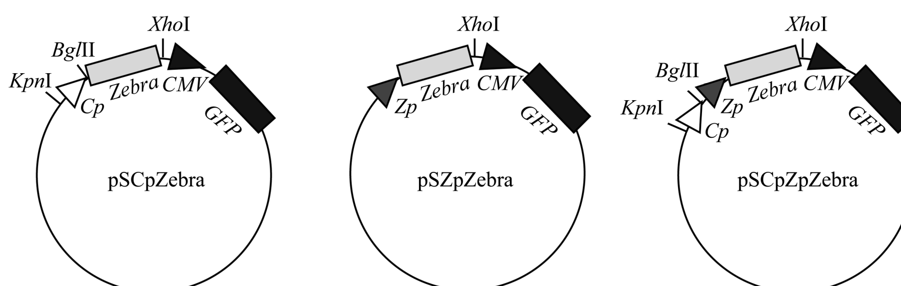

Fig. 1 The three shuttle vectors that were constructed

©Institute of Biochemistry and Cell Biology, SIBS, CAS

was visible as an approximately 30 kb fragment in agarose electrophoresis. These DNA fragments were used to transfect the human embryo kidney cell line HEK293 (Shanghai Cell Bank, Committee on Type Culture Collection, Chinese Academy of Sciences) with Lipofectamine and Plus reagent (Invitrogen, San Diego, USA) according to the manufacturer's instructions. Green fluorescence was observed under a microscope (Nikon) 3 days after transfection. The transfected cells were collected after 10 days resuspended with phosphate-buffer saline (PBS), and frozen and thawed three times to release the virus particle. Cell debris was removed by brief centrifugation and the recombinant adenovirus suspension was stored at –20 °C. The three recombinant adenoviruses, AdvCpZpZebra, AdvCpZebra and AdvZpZebra, were thus constructed. The adenoviruses were further propagated in HEK293 cells.

Cell infection and Zebra protein expression

The EBV-negative cell lines HEK293 and HeLa (Shanghai Cell Bank, Committee on Type Culture Collection, Chinese Academy of Sciences), and the EBV-positive cell line D98-HR1, a hybrid cell line established by the fusion of the human cervix epithelial adenocarcinoma cell line HeLa and the latent EBV-infected lymphoma cell line HR1, were infected with recombinant adenoviruses at different multiplicity of infections (MOIs) for 2 h before fresh medium was added.

The infected cells were collected 72 h post-infection (p.i.), washed with PBS twice, and lysed with sample buffer before being analyzed with SDS-polyacrymide gel electrophoresis (SDS-PAGE) and immunoblotting. Zebra antibodies (Chemicon, Temecula, California, USA) and early antigen-D (EA-D; Novocastra, Newcastle upon Tyne, UK), an EBV early antigen, were used in the immunoblotting.

Cell viability and proliferation assay

For the cell viability assay, the cell suspension of infected D98-HR1 cell (provided by Dr. R. SUN; University of California, Los Angles, USA) or HeLa cell (72 h p.i.) were mixed with an equal volume of 0.4% Trypan blue prepared in PBS, and incubated for 5 min before being checked for the percentage of stained cells using the Nikon microscope. For each treatment, the average percentage of viable cells and standard deviation were calculated from four wells.

For the MTT cell proliferation assay, adenovirus-infected cells were cultured in RPMI 1640 without phenol red for 96 h p.i.. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; purchased from Sigma, Sigma-Aldrich, St. Louis, USA) was added to the infected cells until the final concentration was 0.5 mg/ml. The cells were further incubated for 2 h before an equal volume of 0.04 M HCl in isopropanol was added to solublize the dye. The absorbance was measured at a wavelength of 570 nm, with the background subtraction at 650 nm. The average readings and standard deviations were calculated from the three sets of readings obtained for each treatment. The relative cell proliferation index was calculated by dividing the average reading with the average reading of non-infected cells.

Results

Construction of recombinant viruses

B95-8 cells were treated with TPA and *n*-butyrate to induce EBV lytic replication. Total cellular DNA was used as the template to amplify the *Zebra* gene and viral promoters. Three PCR products with lengths of 1.6, 1.0 and 1.2 kb were obtained, which were the *Zebra* coding sequence with its native promoter *Zp* (*ZpZebra*), the *Zebra* coding sequence only (*Zebra*) and the viral *Cp* promoter (*Cp*), respectively. The PCR products were cloned and verified by sequence analysis (data not shown).

Three recombinant adenoviruses, AdvZpZebra, AdvCpZebra and AdvCpZpZebra, were then constructed as described in the "Materials and Methods". These viruses had the *Zebra* gene expression cassette led by its native promoter *Zp*, the major viral latent promoter *Cp*, or the dual promoter *CpZp*, respectively. They also contained the green fluorescent protein gene led by the *CMV* promoter. The infected cells displayed strong fluorescence under the fluorescence microscope (data not shown). Because these recombinant adenoviruses did not contain the early gene *E1A*, which is essential for virus replication, they could only replicate in *E1A*-transformed cells, such as HEK293. However, this would not impair their ability to infect other cell lines and express the *Zebra* gene there.

Expression of Zebra protein

A protein band with the molecular weight expected for the Zebra protein (42 kDa) was detected in HEK293 cells infected with AdvZpZebra, AdvCpZebra and AdvCpZpZebra when immunoblotted with the anti-Zebra antibody (**Fig. 2**). The expression level of the Zebra protein was similar for all three recombinant viruses. No Zebra protein was detected in HEK293 cells infected with a control adenovirus constructed in the same way, but with only the *GFP*

Downloaded from https://academic.oup.com/abbs/article/37/4/215/126 by guest on 24 April 2024

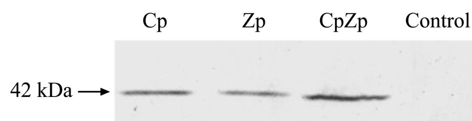

Fig. 2 Immunoblot detection of Zebra protein in HEK293 cells infected with recombinant adenoviruses and the control virus

Cp, AdvCpZebra; Zp, AdvZpZebra, CpZp, AdvCpZpZebra. A band with a molecular weight of 42 kDa, corresponding to the Zebra protein, was observed in Cp, Zp and CpZp, but not in the control.

expression cassette.

Activation of lytic infection

The EBV-positive cell line D98-HR1 was used for the EBV activation experiment. D98-HR1 was selected because, unlike most EBV-positive cell lines, it is highly susceptible to adenovirus infection. As shown in **Fig. 3**, both Zebra and the EBV lytic-phase early antigen EA-D were detected in D98-HR1 cells infected with the three recombinant adenoviruses, while neither of these two proteins was observed in the control adenovirus-infected D98-HR1 cells. The appearance of EA-D is an indication that the lytic replication of the latent EBV has been induced. The expression level of EA-D was correlated with the expression level of Zebra and the virus dose used in the infection (**Fig. 4**). No EA-D was detected at a low viral dose (MOI=2 pfu/cell).

Cell viability assay

To study the effect of *Zebra* expression on cell viability and proliferation, AdvCpZebra was used to infect HeLa (EBV-negative) and D98-HR1 (EBV-positive) cells at the MOI of 5 and 10 pfu/cell, respectively. Cell viability was assayed by Trypan blue staining at 72 h p.i., and the percentages of viable cell were shown in **Fig. 5**. Neither AdvCpZebra nor the control adenovirus caused significant cell death in HeLa cells. D98-HR1 cells were more sensitive to adenovirus infection, especially at high MOI. AdvCpZebra caused significantly more cell deaths than the control virus (P=0.02 and 0.005 for MOI=5 and 10 pfu/cell, respectively).

A similar observation was made in D98-HR1 cells infected with another recombinant adenovirus, AdvCpZpZebra, at various MOI (1, 5 and 25 pfu/cell). MTT assay was used to measure the relative cell viability and proliferation at 96 h p.i. As shown in **Fig. 6**, the virus caused a significant reduction in cell proliferation and viability for all three MOIs (P<0.01 vs. the control), while

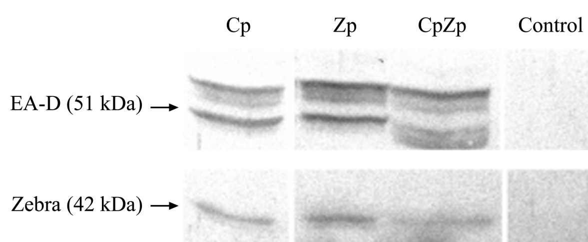

Fig. 3 Immunoblot detection of Zebra and EA-D expression in D98-HR1 cells infected with recombinant adenoviruses and the control virus

Cp, AdvCpZebra; Zp, AdvZpZebra; CpZp, AdvCpZpZebra. Both EA-D protein (51 kDa) and Zebra protein (42 kDa) were observed.

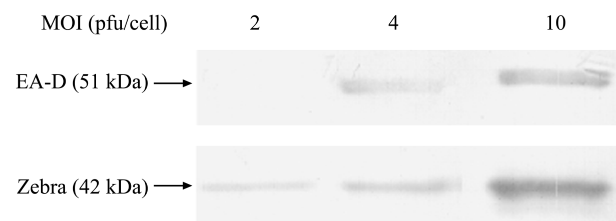

Fig. 4 Immunoblot detection of Zebra and EA-D expression in D98-HR1 cells infected with AdvCpZebra at different Multiplicity of Infection

The EA-D expression correlated with the Zebra expression.

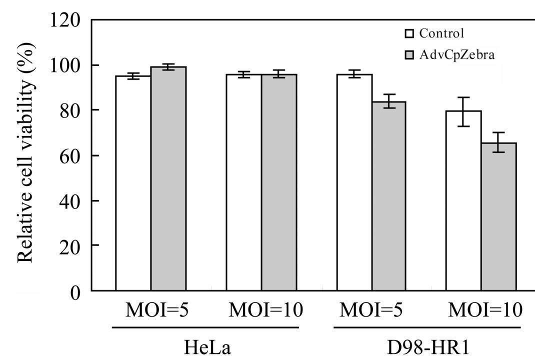

Fig. 5 Cell viability assay of HeLa and D98-HR1 cells infected with AdvCpZebra and the control virus

The error bar indicates the standard deviation from four iterations.

©Institute of Biochemistry and Cell Biology, SIBS, CAS

Downloaded from https://academic.oup.com/abbs/article/37/4/215/126 by guest on 24 April 2024

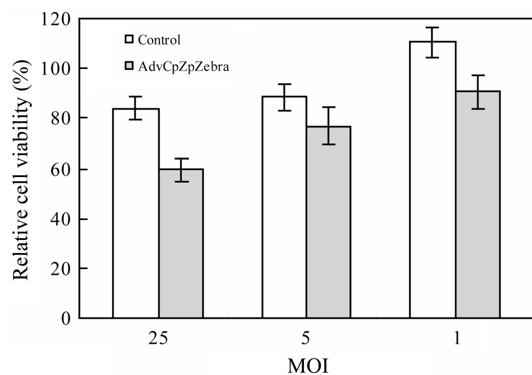

Fig. 6 MTT cell proliferation assay of D98-HR1 cells infected with AdvCpZpZebra

The error bar indicates the standard deviation from three iterations.

the strongest effect was observed at an MOI of 25 pfu/cell.

Discussion

Three recombinant adenoviruses using different EBV promoters, or a combination of promoters, to express the EBV lytic replication activation protein Zebra were constructed and used to induce EBV lytic replication in the EBV-positive cell line D98-HR1. Zebra protein expression was detected in both HEK293 and D98-HR1 cells infected with these viruses. The EBV antigen EA-D was also detected in infected D98-HR1 cells, which strongly indicates that viral lytic replication was induced.

Cp and *Zp* are EBV promoters that are more active in EBV-positive cells than in normal cells. *Cp* is the major promoter in EBV-latent infections [15], while *Zp*, the specific promoter of the *Zebra* gene, is auto-regulated by Zebra [16]. These promoters may facilitate the specific expression of Zebra in EBV-positive cells. A high Zebra expression level in the EBV-negative HEK293 cells (**Fig. 2**) is not unexpected as HEK293 is a cell line that allows full replication of these recombinant adenoviruses. In EBV-positive D98-HR1 cells, *Cp*, *Zp* and *CpZp*, the dual promoter combining two promoters in tandem, did not show significant differences in the *Zebra* expression level.

The cell viability assay showed that recombinant adenoviruses had little effect on the EBV-negative HeLa cells, while significantly reducing the viability of EBV-positive D98-HR1 cells. More cell lines, either EBV-positive or EBV-negative, need to be tested to further confirm the EBV-associated *Zebra* expression and cell-killing capability of these recombinant adenoviruses.

EBV is believed to be associated with many types of human cancer, among which NPC has the most consistent association. As one of the common cancers in southern China and Southeast Asia, NPC has several etiological factors, including genetic susceptibility [17], food and environmental factors [18] and EBV infection [2–5]. EBV is found in virtually all cases of non-keratinizing NPC, the most common subtype of NPC (80% of all NPC cases), and EBV genomes are detectable in almost all cells of this cancer subtype [19]. The specific expression of viral proteins and the activation of the latent EBV may offer an attractive approach for the control of such cancers. Because most people with EBV-associated cancer have a high titer of antibodies against EBV, viruses produced by lytic replication should be quickly controlled by the host immune system. The application of antiviral drugs together with these recombinant viruses will also help to limit the spread of the viruses. Certain types of antiviral drugs, such as gancyclovir, while inhibiting virus production, may also enhance the cell-killing effect through the bystander effect, as thymidine kinases produced by the activated EBV will convert them into a form that is toxic for cells.

References

1 Hsu JL, Glaser SL. Epstein-Barr virus-associated malignancies: Epidemiologic patterns and etiologic implications. Crit Rev Oncol Hematol, 2000, 34(1): 27–53

2 Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer, 2004, 4(10): 757–768

3 Niedobitek G. Epstein-Barr virus infection in the pathogenesis of nasopharyngeal carcinoma. Mol Pathol, 2000, 53(5): 248–254

4 Spano JP, Busson P, Atlan D, Bourhis J, Pignon JP, Esteban C, Armand JP. Nasopharyngeal carcinomas: An update. Eur J Cancer, 2003, 39(15): 2121–2135

5 Raab-Traub N. Epstein-Barr virus in the pathogenesis of NPC. Semin Cancer Biol, 2002, 12(6): 431–441

6 Fukuda M, Kurosaki W, Yanagihara K, Kuratsune H, Sairenji T. A mechanism in Epstein-Barr virus oncogenesis: Inhibition of transforming growth factor-beta 1-mediated induction of MAPK/p21 by LMP1. Virology, 2002, 302(2): 310–320

7 Damania B. Oncogenic γ-herpesviruses: Comparison of viral proteins involved in tumorigenesis. Nat Rev Microbiol, 2004, 2(1): 656–668

8 Israel BF, Kenney SC. Virally targeted therapies for EBV-associated malignancies. Oncogene, 2003, 22(33): 5122–5130

9 Flemington EK. Herpesvirus lytic replication and the cell cycle: Arresting new developments. J Virol, 2001, 75(10): 4475–4481

10 Ragoczy T, Miller G. Role of the Epstein-Barr virus RTA protein in activation of distinct classes of viral lytic cycle genes. J Virol, 1999, 73(12): 9858–9866

11 Francis A, Ragoczy T, Gradoville L, Heston L, El-Guindy A, Endo Y, Miller G. Amino acid substitutions reveal distinct functions of serine 186 of the ZEBRA protein in activation of early lytic cycle genes and synergy with the

Downloaded from https://academic.oup.com/abbs/article/37/4/215/126 by guest on 24 April 2024

Epstein-Barr virus R transactivator. J Virol, 1999, 73(6): 4543–4551
12 Ragoczy T, Heston L, Miller G. The Epstein-Barr virus RTA protein activates lytic cycle genes and can disrupt latency in B lymphocytes. J Virol, 1998, 72(10): 7978–7984
13 Feng WH, Westphal E, Mauser A, Raab-Traub N, Gulley ML, Busson P, Kenney SC. Use of adenovirus vectors expressing Epstein-Barr virus (EBV) immediate-early protein BZLF1 or BRLF1 to treat EBV-positive tumors. J Virol, 2002, 76(21): 10951–10959
14 He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA, 1998, 95(5): 2509–2514
15 Woisetschlaeger M, Yandava CN, Furmanski LA, Strominger JL, Speck SH. Promoter switching in Epstein-Barr virus during the initial stages of infection of B lymphocytes. Proc Natl Acad Sci USA, 1990, 87(5): 1725–1729
16 Kraus RJ, Mirocha SJ, Stephany HM, Puchalski JR, Mertz JE. Identification of a novel element involved in regulation of the lytic switch BZLF1 gene promoter of Epstein-Barr virus. J Virol, 2001, 75(2): 867–877
17 Feng BJ, Huang W, Shugart YY, Lee MK, Zhang F, Xia JC, Wang HY *et al*. Genome-wide scan for familial nasopharyngeal carcinoma reveals evidence of linkage to chromosome 4. Nat Genet, 2002, 31(4): 395–399
18 Yu MC, Yuan JM. Epidemiology of nasopharyngeal carcinoma. Semin Cancer Biol, 2002, 12(6): 421–429
19 Niedobitek G, Agathanggelou A, Nicholls JM. Epstein-Barr virus infection and the pathogenesis of nasopharyngeal carcinoma: Viral gene expression, tumour cell phenotype, and the role of the lymphoid stroma. Semin Cancer Biol, 1996, 7(4): 165–174

Edited by
Zu-Xun GONG

Downloaded from https://academic.oup.com/abbs/article/37/4/215/126 by guest on 24 April 2024

©Institute of Biochemistry and Cell Biology, SIBS, CAS