

Carboxyl terminal activating region 3 of latent membrane protein 1 encoded by the Epstein-Barr virus regulates cell proliferation and protein expression in NP69 cells

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Received November 10, 2017; Accepted April 23, 2018

DOI: 10.3892/mmr.2019.10859

Abstract. In the present study, the mechanism by which carboxyl terminal activating region 3 (CTAR3) of latent membrane protein 1 (LMP1), encoded by the Epstein-Barr virus, regulated cell proliferation and protein expression was investigated in the nasopharyngeal epithelial cell line NP69. The deletion mutant LMP1 (LMP1^{Δ232-351}; amino acid residues including 232-351 codons in CTAR3 deleted) was generated by polymerase chain reaction. An NP69-LMP1^{Δ232-351} cell line was established by retroviral infection. Finally, cell proliferation and protein expression of NP69 cells expressing LMP1^{Δ232-351} were examined using a cell growth curve and western blot analysis. The results demonstrated: i) The proliferation of NP69-LMP1^{Δ232-351} cells was significantly decreased compared with cells expressing wild type LMP1 (LMP1^{WT}; n=3; P<0.05); ii) 17 proteins exhibited differential protein expression (>2-fold change) in NP69-LMP1^{Δ232-351} cells compared with NP69-LMP1^{WT} cells; and iii) LMP1^{WT} was involved in activating the Janus

kinase 3 (JAK3) promoter and regulating the expression of JAK3 protein, while LMP1^{Δ232-351} was almost defective in ability to activate the JAK promoter. These results suggested that LMP1-CTAR3 may be an important functional domain for regulating cell proliferation and protein expression in nasopharyngeal epithelial cells.

Introduction

Epstein Barr-virus (EBV) is a highly prevalent γ herpes virus in humans, and was also the first identified human tumor-associated virus (1). EBV is frequently implicated in the etiology of a number of malignancies, including nasopharyngeal carcinoma (NPC) (1). NPC, particularly poorly differentiated or undifferentiated NPC, is closely associated with EBV. Latent membrane protein 1 (LMP1) is known to be a oncogenic protein encoded by the EBV genome, as it has an important role in EBV-mediated B-cell proliferation and immortalization (2); however, the mechanism of LMP1-mediated epithelial cell transformation remains unclear. LMP1 is a 386 amino acid transmembrane glycoprotein, which consists of a short cytoplasmic N-terminal domain (residues 1-23), six transmembrane domains (residues 24-186), and a long cytoplasmic C-terminal domain (residues 187-386), which is known as the carboxyl terminal activation region (CTAR) (3). Currently, three CTARs have been reported, CTAR1 (residues 194-232), CTAR2 (residues 351-386) and CTAR3 (residues 275-330) (4). CTAR1 engages tumor necrosis factor receptor-associated factors to induce low-level nuclear factor- κ B (NF- κ B) activation (5). CTAR2 interacts with TRAF3 death domain protein to mediate high-level NF- κ B activation and also induces c-Jun N-terminal kinases/activator protein-1 (AP-1) activation (6). Gires *et al* (7) first reported the CTAR3 of LMP1 and confirmed the region was associated with the JAK3/signal transducer and activator of transcription (STAT) signaling pathway; however, its function in epithelial cells requires further analysis.

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Key words: latent membrane protein 1, carboxyl terminal activating region 3, NP69, cell proliferation, proteome, Janus kinase 3

Materials and methods

Plasmids. NF- κ B luciferase (LUC) reporter and β -galactosidase plasmids were received from Dr David Goeddel (Tularik, Inc., San Francisco, CA, USA). AP-1 LUC reporter (with four AP-1 sites) was received from Dr Zhi-Gang Dong (University of Minnesota, Austin, MN, USA). pLNSX retroviral vector, pLNSX-LMP1^{WT} retroviral vector (wild type with the full-length LMP1 gene) and pGL2 plasmids were received from Dr Liang Cao (University of Hong Kong, Hong Kong SAR, China).

Cell lines. The SV40-immortalized nasopharyngeal epithelial cell line NP69 was a generous gift from Dr Sai Wah Tsao (University of Hong Kong). NP69 cells were cultured in serum-free keratinocyte medium (K-SFM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in humidified 5% (v/v) CO₂ atmosphere at 37°C. Retrovirus packaging cell line PA317, immortalized lymphocyte cells and 293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and routinely maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 15% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.).

Reagents and primers. The mouse anti-human monoclonal antibody S12 for LMP1 (1:50) obtained from a hybridoma was a generous gift from Dr Liang Cao (University of Hong Kong, SAR, China). Immobilized pH gradient (IPG) strip (pH 3-10NL, 24 cm) were obtained from GE Healthcare (Chicago, IL, USA). Polymerase chain reaction (PCR) primers (Table I) were designed using Primer5 software (version 5.00; Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Invitrogen (Thermo Fisher Scientific, Inc.).

Reorganization of the pLNSX-LMP1^{Δ232-351} plasmid and pGL2/Janus kinase 3 (JAK3)-LUC plasmid. To construct an pLNSX-LMP1^{Δ232-351} plasmid expressing a product with deleted amino acid residues at positions 232 to 351 in the CTAR3 region, plasmid pLNSX-LMP1^{WT} with full-length LMP1^{WT} gene as a template and four primers (p1, 5'CTCGGCCTCTGAGCTATTCC3'; p2, 5'GCCGCCATGGGCTCCACTCACTCACGAGCAG3'; p3, 5'AGTGGAGCCCATGGC GGCGGTGACCCA3' and p4, 5'CGAGAAGCGAACTGATTGGT3') were put into the PCR instrument (Eppendorf, Hamburg, Germany). The 50 μ l PCR reaction was carried out using a PCR amplification kit (Promega (Beijing) Biotech Co., Ltd, Beijing, China) according to the manufacturer's protocol. PCR was performed for 30 cycles and consisted of denaturation at 94°C for one minute, annealing at 58°C for 1 min and extension at 72°C for 1 min. The PCR product and the pLNSX retroviral vector containing XbaI and HindIII sites were mixed in 10 μ l PCR reaction system at 1:1 ratio. The connection reaction was run overnight at 16°C. The pLNSX-LMP1^{Δ232-351} was confirmed by DNA sequencing. In addition, to construct the JAK3 LUC reporter plasmid, genomic DNA of immortalized lymphocyte cells was used as the PCR template with upstream primer (5'CCGCTCGAGGTGCCCACTCACACATGCTACAGAT3', the *Xho*I site denoted by bold font) and downstream primer (5'CCCAAGCTTAGAGGAAAGTCCCACTCGGCTCCTT3', the *Hind*III site denoted by bold font). PCR was

performed for 35 cycles consisting of denaturation at 94°C for one minute, annealing at 63°C for 1 min and extension at 72°C for 30 sec. Subsequently, the PCR product was digested with *Hind*III and *Xho*I and cloned into the luciferase reporter plasmid pGL2. The pGL2/JAK3-LUC plasmid was obtained and confirmed by DNA sequencing.

Retrovirus-mediated gene transfer and detection of the expressed proteins. PA317 cells were transfected with pLNSX-LMP1^{WT} and pLNSX-LMP1^{Δ232-351} at 300 ng/well, to produce amphotropic retroviruses at a density of 5x10⁴ cells per well in 6-well plates. After 24 h of transfection, the transfected PA317 cells were selected with G418 (500 mg/ml, Invitrogen; Thermo Fisher Scientific, Inc.). After 2-3 weeks, the resistant cells were collected and cultured as the virus-producing cell lines. NP69 cells, which were inoculated onto 6-well plates in triplicate at 5x10⁴ cells/well, were transduced with Rv-LMP1^{WT}, Rv-LNSX-LMP1^{Δ232-351} retroviruses (MOI=30). After 48 h, the successfully infected cells were selected by G418 (400 μ g/ml) for 2 weeks, and the resistant clones were pooled and designated as NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351} cells. The stable expression of LMP1 in the resistant clones was determined by western blotting and immunofluorescence assay with LMP1 antibody S12.

Immunofluorescence assay. Cell slides were prepared, fixed with methanol and acetone (1:1) at 4°C for 30 min, washed, dried, and labeled with anti-LMP1 monoclonal antibody S12 (1:50) for 1 h at 37°C. After washing, the cells were incubated with a FITC-labeled goat anti-mouse secondary antibody (1:500; cat. no. A-11029; Zymed; Thermo Fisher Scientific, Inc.) for 1 h at 37°C, washed and then oil sealed and observed under a fluorescence microscope at 778 nm (EVOS[®] FL Auto; Thermo Fisher Scientific, Inc.).

Cell growth curve analysis. For growth curve analysis, transduced NP69 cells were seeded onto 96-well plates in triplicate wells at 1x10⁴ cells/well at 24 h after viral infection. The number of viable cells was determined every 48 h using the MTT method. After incubation with MTT in a humidified 5% (v/v) CO₂ atmosphere at 37°C incubator for 4 h, the cell culture supernatant was removed, 150 μ l of DMSO was added to fully dissolve the crystals and the OD value of each well was measured at a wavelength of 490 nm using an ELX800 microplate reader (BioTek China, Beijing, China). Growth curves were produced by plotting the mean and standard deviation of three independent experiments.

Soft agar clone formation assay. To determine the anchorage-independence ability of NP69 cells, LMP1 cells that had been transduced for 24 h were seeded into semisolid agar K-SFM medium [base layer, 0.6% (w/v); upper layer, 0.3% (w/v)] at a density of 5x10⁴ cells per well in 6-well plates. Three independent experiments with duplicate wells for each cell line were performed. After 3-4 weeks of incubation at 37°C with 5% (v/v) CO₂, the number and size of colonies (\geq 50 cells was classed as one colony) were observed under an inverted microscope (TS100; Nikon Corporation, Tokyo, Japan), and 10 low power fields (x4) were randomly selected for each group. The experiments were conducted in triplicate.

Table I. Primer sequences used in fluorescent reverse transcription-quantitative polymerase chain reaction.

Primers	Sequence (5'-3')	Product size (bp)
Ribosomal protein P0	Sense AAGGCTGTGGTGCTGATG Antisense GTCCTCCTTGGTGAACACA	132
Annexin A2	Sense ATCTCTATGACGCTGGAGTGAA Antisense GGGCTGTAACCTCTTGTACCTATCA	121
Heterogeneous nuclear ribonucleoprotein A/B	Sense CTGGATGGCCGTGTCATT Antisense GCCTCAATCTCCCCAAACT	143
Isocitrate dehydrogenase 3	Sense TGCAGAGTATCAAGCTCATCAC Antisense TAGAAAAAGCCCATCTGACATC	143
G protein	Sense GGGTCACTCCCACCTTTGTTAG Antisense TCAGCACATCCTTGGTATGG	149
β -actin (internal control)	Sense ACCGTGGAGAAGAGCTACGA Antisense GTACTTGCGCTCAGAAGGAG	309

Preparation of the total protein sample. Harvested cells were washed twice with ice-cold PBS, and lysed in lysis buffer [7 mol/l urea, 2 mol/l thiourea, 2% (v/v) NP-40, 1% (v/v) Triton X-100, 100 mmol/l dithiothreitol (DTT), 5 mmol/l PMSF, 4% (w/v) CHAPS, 0.5 mmol/l EDTA, 40 mmol/l Tris, 1 mg/ml DNase I]. The cell lysates were incubated at 4°C for 30 min and then centrifuged at 4°C and 21,130 x g for 10 min. The supernatant constituted the total protein solution. The concentration of the total proteins was assayed with a 2D Quantification kit (GE Healthcare).

IPG-2D PAGE and image analysis. IPG-2D PAGE was performed according to the manufacturer's protocols (GE Healthcare). Protein samples (1.0 mg) were diluted to 450 μ l with rehydration solution [8 mol/l urea, 2% (w/v) CHAPS, 0.5% (v/v) pH 3-10 IPG buffer and trace bromophenol blue], and applied for isoelectric focusing (IEF) using Immobiline IPG strips (pH 3-10; L 240x3x0.5 mm). The strips were rehydrated at 30 V for 14 h and proteins were focused successively for 1 h at 500 V, 1 h at 1,000 V and 8.5 h at 8,000 V to produce a total of 69,920 Vh on an IPGphor (Amersham Biosciences, Uppsala, Sweden). Following IEF, the IPG strips were equilibrated for 15 min at room temperature in a buffer containing 50 mM Tris-HCl (pH 8.8), 30% (v/v) glycerol, 6 M urea, 2% (w/v) SDS and 1% (w/v) DTT, followed by further treatment in a similar buffer [containing 2.5% (w/v) iodoacetamide instead of DTT] for 15 min, and then directly applied on to 12.5% (w/v) homogeneous SDS-PAGE gels for electrophoresis using a the Ettan DALT II system (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocols. The separated proteins were visualized after 13 h Coomassie Brilliant Blue G-250 staining at room temperature. The stained 2D gels were scanned with MagicScan software (version 6.0; Kenxen Limited, Hong Kong, SAR, China) on an Imagescanner (Amersham Biosciences, Uppsala, Sweden) with 300 DPI resolution. The scanned data were then analyzed using PDQuest 2D gel analysis software (version 7.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA). To ensure experiment reproducibility, the 2D gel of each cell line was repeated in triplicate. The gel spot pattern of each gel was summarized

in a standard following spot matching. Thus, one standard gel for each cell line was established. The criteria to determine differential protein spots were that spot intensity increased or decreased more than two-fold between the comparison groups. Statistical analysis was performed using SPSS, version 18.0 (SPSS, Inc. Chicago, IL, USA).

Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). A total of 39 differential protein spots were excised from preparative 2D gels using biopsy punches and transferred to a 1.5 ml siliconized Eppendorf tube. Each spot was first washed with 50 μ l deionized water 3 times, each time for 2 min. A further 50 μ l of decolorizing solution (100 mmol/l NH_4HCO_3 , 30% CAN; 1:1) was added at 37°C for 30 min. The spot was then repeatedly rinsed with deionized water until the color had completely faded. A 300 μ l volume of 100% CAN was added to dehydrate the sample before it was drained. A 5 μ l volume of trypsin working solution (0.02 μ g/ μ l) was added to each tube to digest the sample at 4°C for 45 min. After the solution had been completely absorbed by micelles, 30 μ l of NH_4HCO_3 (40 mmol/l) was added and the sample was incubated at 37°C overnight. The supernatant was transferred to a new tube, and 30 μ l extraction liquid (60% CAN, 5% TFA; 1:1) was added to the original tube, the extraction was repeated twice at 37°C for 60 min. After extraction and enzymolysis the supernatants were combined and lyophilized to prepare the protein sample. The protein samples were analyzed by Applied Biosystems Voyager System 4307 MALDI-TOF mass spectrometer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The parameters were set up as follows: Positive ion-reflector mode, accelerating voltage 20 kV, grid voltage 64.5%, mirror voltage ratio 1.12, N_2 laser wavelength 337 nm, pulse width 3 nsec, the number of laser shots 50, acquisition mass range 1,000-3,000 Da, delay 100 nsec and vacuum degree 4×10^{-7} Torr. A trypsin-fragment peak served as internal standard for mass calibration. A list of the corrected mass peaks was termed the peptide mass fingerprint (PMF). Proteins were identified from PMF data by searching the UniProt database (<http://www.uniprot.org/uniprot>) using the software MASCOT

(version 2.5.1, Matrix Science, Ltd., London, UK). Subcellular location and function information was derived from the NCBI database (<http://www.ncbi.nlm.nih.gov>).

Protein extraction and western blot analysis. Cells were extracted in lysis buffer (0.5% Nonidet P-40, 5% sodium deoxycholate, 50 μ M NaCl, 10 μ M Tris-HCl, pH 7.5, 1% bovine serum albumin) and centrifuged at 4°C and 18,407 x g for 15 min. The supernatant was mixed with 2X loading buffer and boiled for 5 min, and then the samples were separated via 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% fat-free milk at room temperature for 1 h, incubated at 4°C overnight with antibodies S12 for LMP1 (1:50), anti- β -actin (1:10,000; cat. no. A1978; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), anti-G protein (1:500; cat. no. 371818; Sigma-Aldrich; Merck KGaA) and anti-heterogeneous nuclear ribonucleoprotein A/B (1:500; cat. no. sc-376411; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), washed, and then incubated with peroxidase-conjugated secondary antibody (1:2,000; cat. no. sc-11001; Santa Cruz Biotechnology, Inc.). Immune complexes were detected using an Amersham ECL Western Blotting Detection kit (Amersham Pharmacia Biotech, Little Chalfont, UK) and gel imaging analysis (Cool Imager; Viagene Biotech, Inc). To confirm the expression levels of each protein examined in LMP1-transfected cells, western blotting for each protein was performed in triplicate.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The gene expression of the differential proteins was quantitated by RT-qPCR using Roche Light Cycler system (Roche Diagnostics GmbH, Mannheim, Germany) and SYBR premix Ex Taq kit (Takara Bio, Inc., Otsu, Japan). The expression levels of β -actin served as an internal control. Total cellular RNA was isolated from NP69-LMP1^{WT} and NP69-LMP1 ^{Δ 232-351} cells using TRIzol[®] (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA integrity was observed by electrophoresis with 1% agarose gel containing ethidium bromide. A total of 2 μ g DNase-treated RNA underwent RT to produce cDNA and 1 μ l RT product was used to amplify gene fragments. First-strand cDNA was synthesized from DNase-treated total RNA with oligo-dT primer and Super-Script II reverse transcriptase (Takara Bio, Inc.) for 60 min at 42°C and 10 min at 72°C, followed by qPCR amplification using the corresponding specific primers (Table I). The qPCR cycling conditions were as follows: 95°C for 3 min followed by 50 cycles of 95°C for 30 sec, 62°C (ribosomal protein P0 and isocitrate dehydrogenase 3) or 65°C (annexin A2, heterogeneous nuclear ribonucleoprotein A/B and G protein) for 30 sec, and then 72°C for 30-40 sec. The relative fold change method (8) was used to determine the relative quantitative gene expression for each gene studied compared with the β -actin control. The relative fold change or relative gene expression value $2^{-\Delta\Delta Cq}$, where $\Delta\Delta Cq = [(Cq_{\text{Target[LMP1]}^{\text{WT}}}] - Cq_{\beta\text{-actin[LMP1]}^{\text{WT}}}] - (Cq_{\text{Target[LMP1]}^{\Delta 232-351}}] - Cq_{\beta\text{-actin[LMP1]}^{\Delta 232-351}}]$. $Cq_{\text{Target[LMP1]}^{\text{WT}}}$ = quantification cycle of the target gene examined in the LMP1-expressing cells; $Cq_{\beta\text{-actin[LMP1]}^{\text{WT}}}$ = quantification cycle of the β -actin gene in the LMP1-expressing cells; $Cq_{\text{Target[LMP1]}^{\Delta 232-351}}$ = quantification cycle of the target gene examined in the LMP1 ^{Δ 232-351}-expressing

Table II. Cell culture transformation analysis of NP69-pLNSX, NP69-LMP1^{WT} and NP69-LMP1 ^{Δ 232-351}.

Cells	Foci-forming number
NP69-LMP1 ^{Δ232-351}	88 \pm 7 ^a
NP69-LMP1 ^{WT}	256 \pm 14
NP69-pLNSX	3 \pm 1

^aP<0.05 vs. NP69-LMP1^{WT}. LMP1, latent membrane protein 1; WT, wild type; LMP1 ^{Δ 232-351}, mutant type LMP1.

cells; and $Cq_{\beta\text{-actin[LMP1]}^{\Delta 232-351}}$ = quantification cycle of the β -actin gene in the LMP1 ^{Δ 232-351}-expressing cells. RT-qPCR analyses independently were performed in triplicate.

Transcription activity analysis. For each transfection, 293 cells were seeded into 6-well dishes at 1x10⁵ cells/well. The indicated amounts (500, 150, 300, 450 or 600 ng/well) of pLNSX, pLNSX-LMP1 or pLNSX-LMP1 ^{Δ 232-351} plasmids were co-transfected with β -gal (200 ng/well) and reporter plasmids (200 ng/well) of NF- κ B, AP-1 or JAK3, respectively. Vector pLNSX was supplemented to a total amount of 1 μ g DNA for every well. Transfection was conducted with transfection reagent Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cell lysates were collected 24 h later and were examined with a luciferase assay system (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol, to obtain the relative activities of the promoter. Relative LUC values were calculated as the ratio of LUC vs. β -galactosidase activity. A total of three independent experiments were performed, and each experiment was performed in triplicate.

Statistical analysis. Data are presented as the mean \pm standard deviation for \geq 3 separate experiments. Statistical analyses were carried out with SPSS (version 10.01; SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed using a Student's t-test or one-way analysis of variance followed by the Tukey multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of pLNSX-LMP1 ^{Δ 232-351} and pGL2/JAK3-LUC plasmids. To investigate the role of the CTAR3 binding site in LMP1-mediated JAK3 signaling, mutant LMP1 ^{Δ 232-351}, LMP1 with amino acid residue deletion from 232 to 351, was constructed as described, and a JAK3 promoter reporter system was generated using qPCR. The construction of these plasmids was confirmed by enzyme digestion and agarose gel electrophoresis (Fig. 1). The resultant sequence was fully verified by sequencing (data not shown).

Lack of LMP1 CTAR3 reduces the proliferation and growth of NP69 cells. In the present study, the two cell lines, NP69-LMP1^{WT} and NP69-LMP1 ^{Δ 232-351} (Fig. 2A) were established. Then, the expression levels of LMP1 in NP69-LMP1^{WT}

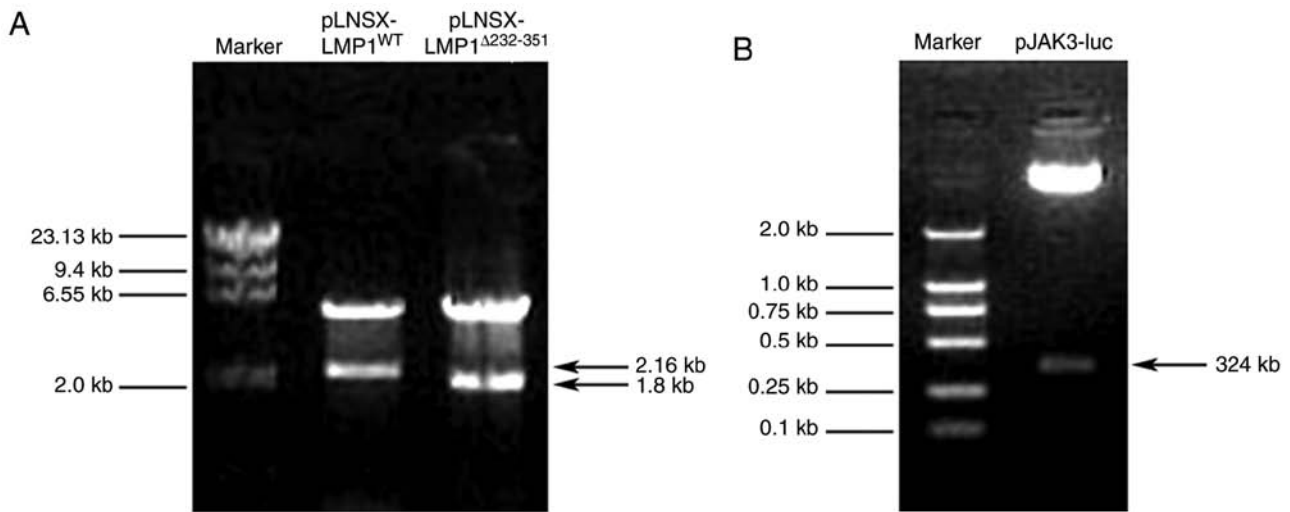


Figure 1. Recombination plasmid identification via enzyme digestion. (A) Recombinant pLNSX-LMP1^{Δ232-351} plasmid was confirmed by *Hind*III and *Xba*I restrictive enzyme digesting analysis. (B) pJAK3-luc plasmid was digested by *Hind*III and *Xho*I restrictive enzyme. The enzyme digestion products were visualized by 1.0% agarose gels stained with ethidium bromide. LMP1, latent membrane protein 1; WT, wild type; LMP1^{Δ232-351}, mutant type LMP1; JAK3, Janus kinase 3.

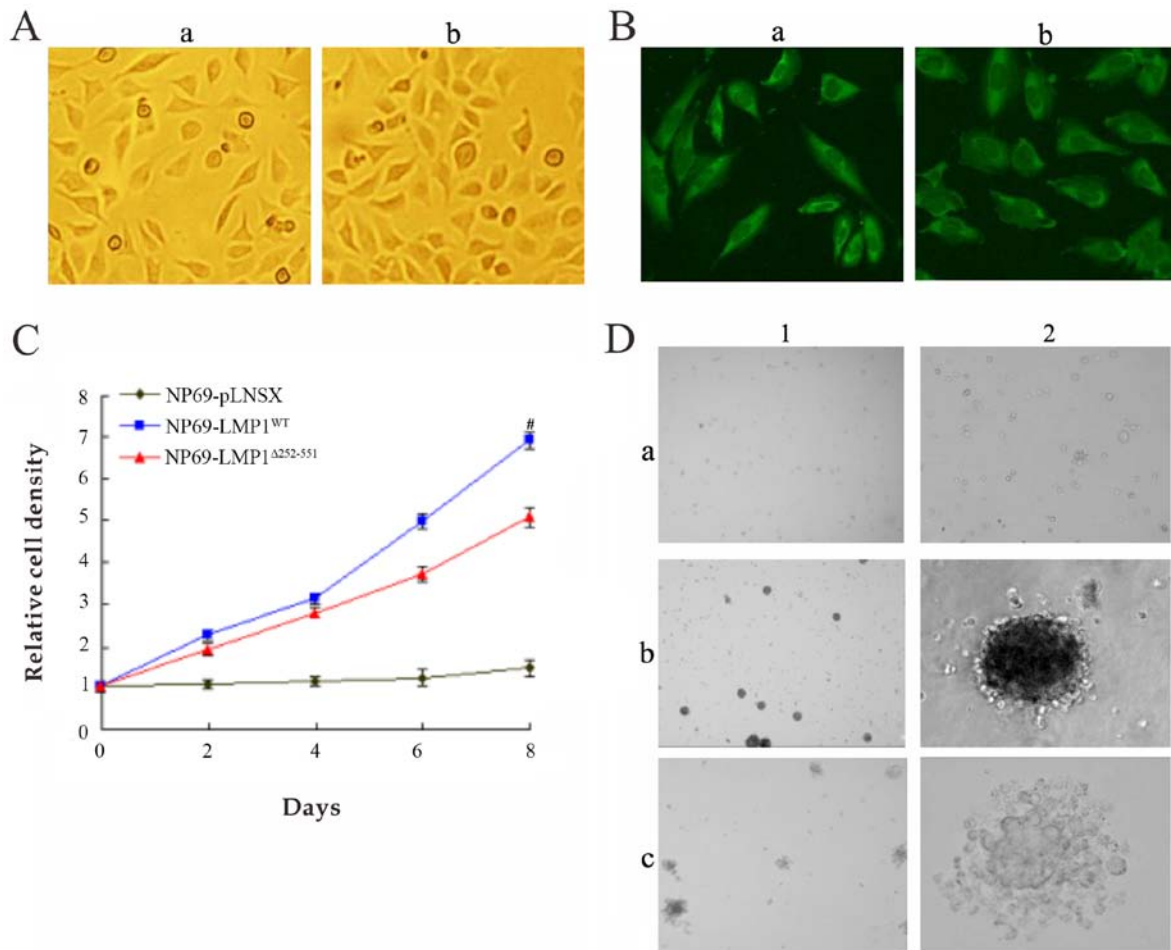


Figure 2. Biological properties of NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351} cell lines (magnification x400). (A) Morphologies of LMP1-expressed NP69 cells. (A-a) NP69-LMP1^{WT} exhibited elongated and fibroblast-like shape. (A-b) NP69-LMP1^{Δ232-351} exhibited similar with fibroblast-like shape. (B) Expression of LMP1 in NP69 cells was detected by immunofluorescence. (B-a) NP69-LMP1^{WT} revealed green fluorescence, demonstrating LMP1 protein expression within the cellular membrane and cytoplasm. (B-b) NP69-LMP1^{Δ232-351} also exhibited LMP1 protein expression. (C) Proliferation of NP69 cell lines expressing LMP1^{WT} and LMP1^{Δ232-351}. The NP69-LMP1^{WT} cells proliferated faster than NP69-LMP1^{Δ232-351} cells. (D) Colonies of NP69 cells in soft agar. There was a marked difference in size and morphology of the soft agar clones between NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351} cells. (D-a1, a2) NP69-pLNSX cells form very few colonies in soft agar. (D-b1, b2) The NP69-LMP1^{WT} soft agar clones were larger, less compact in organization and more irregular in shape. (D-c1, c2) The NP69-LMP1^{Δ232-351} clones were smaller, closely packed and round in shape. Values are presented as the mean ± standard deviation (n=3, #P<0.05 vs. NP69-LMP1^{Δ232-351}). LMP1, latent membrane protein 1; WT, wild type; LMP1^{Δ232-351}, mutant type LMP1.

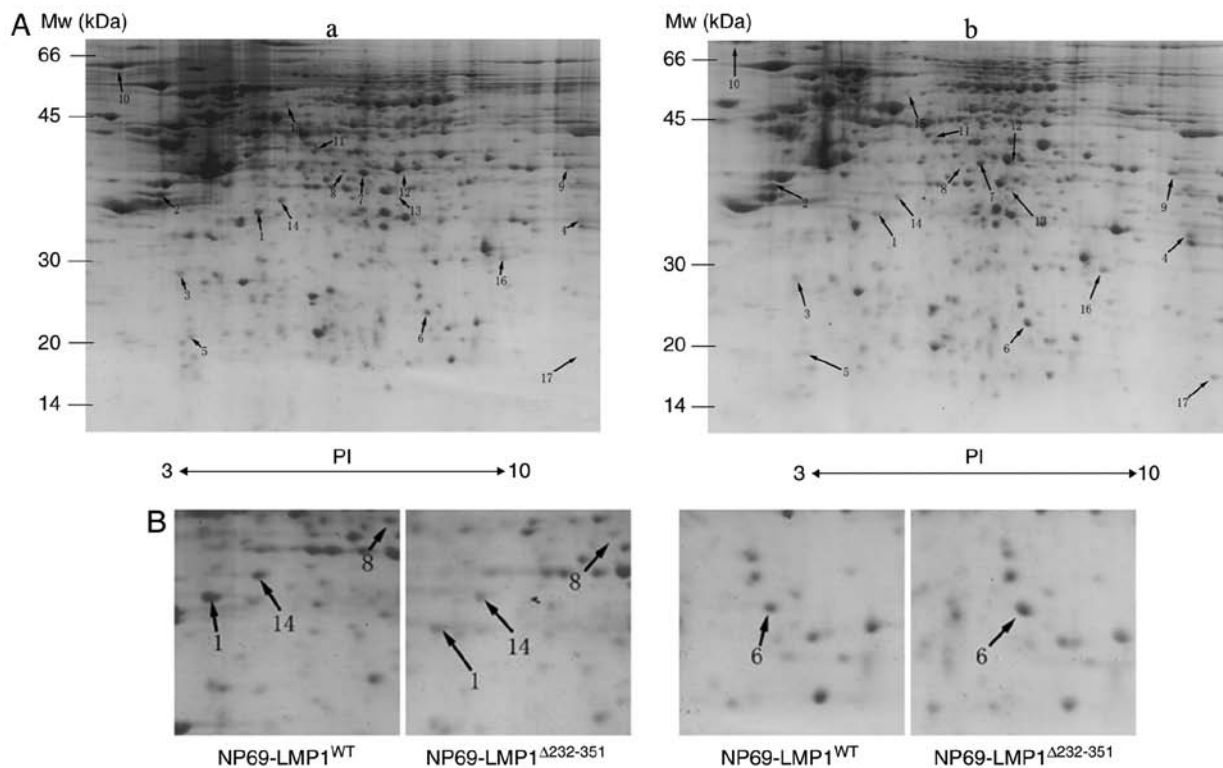


Figure 3. Immobilized pH gradient 2D electrophoresis of NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351} cells. (A) Arrows mark the differential protein spots. (A-a) A representative 2D gel map of NP69-LMP1^{WT} revealed 17 notable differential protein spots compared with (A-b) NP69-LMP1^{Δ232-351}. (B) Protein spots indicated by arrow were seen in both NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351}. LMP1, latent membrane protein 1; WT, wild type; LMP1^{Δ232-351}, mutant type LMP1.

and NP69-LMP1^{Δ232-351} cells were detected by immunofluorescence with antibody against LMP1 S12 (Fig. 2B). In addition, the growth curves of NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351} ($n=3$, $P<0.05$; Fig. 2C) were examined. NP69-LMP1^{WT} cells exhibited a relatively faster growth rate with ~ 7 -8 population doublings by day 8 compared with in control (NP69-pLNSX) cells. Conversely, NP69-LMP1^{Δ232-351} cells only exhibited a growth rate of ~ 4 -5 population doublings by day 8 ($P<0.05$ vs. NP69-LMP1^{WT}). The anchorage-independent growth ability of NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351} cells was also compared. LMP1^{WT} and LMP1^{Δ232-351} expression induced anchorage-independent growth in NP69 cells; however, the cloning efficiency of NP69-LMP1^{WT} cells (256 ± 14 clones) was ~ 3 -fold higher than that of the NP69-LMP1^{Δ232-351} cells (88 ± 7 clones; $n=3$, $P<0.05$; Table II). In addition to the difference in cloning efficiency, there was a marked difference in size and morphology of the soft agar clones between NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351} cells. The NP69-LMP1^{WT} soft agar clones were larger, less compact in organization and more irregular in shape, while the NP69-LMP1^{Δ232-351} clones were smaller, closely packed and round in shape (Fig. 2D). These results suggested that the CTAR3 of LMP1 has an important role on the proliferation and growth of NP69 cells.

Differential protein expression identified using IPG-2D PAGE in NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351} cell lines. In the present study, two reproducible 2D gels for each transduced NP69 cell line were obtained. In the pH range 3-10, there were $1,088\pm 43$ and $1,142\pm 46$ protein spots observed in NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351}, respectively. For the

matched counter-spots, the same position of spots was identified in the two images, which greatly facilitated the following comparison, as presented in magnified views of the 2D gel map. These differential protein spots were marked with arrows (Fig. 3A). Magnified regions of the gels revealed differential expression proteins (Fig. 3B). The quantification of the protein spots, analyzed by PDQuest software, revealed that some protein spots exhibited variable expression levels in the two cell lines, as indicated by staining intensities. A total of 17 protein spots exhibited >2 -fold change in NP69-LMP1^{Δ232-351}-expressing cells compared with NP69-LMP1^{WT}-transduced cells.

Identification of differential protein spots. Following spot excising and tryptic digestion, identification of the protein of interest was performed using MALDI-TOF MS. The expectation value for proteins was determined via PMF by using the MASCOT program. The 17 differential protein spots between NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351} were identified with details summarized in Table III; 8 upregulated and 9 downregulated proteins were associated with structural proteins, metabolic enzymes, repair of DNA damage, energy and electron transport, transcription and translation, molecular chaperone, immunoregulation and calcium-binding, according to UNIPROT and NCBI database.

Validation of the results of partial identified proteins by RT-qPCR and western blotting. The mRNA expression levels of differentially expressed proteins were confirmed by RT-qPCR analysis (data not shown). The results of RT-qPCR analysis coincided with the data of the 2D gel. Within

Table III. Identified differential protein spots between NP69-LMP1^{Δ232-351} and NP69-LMP1^{WT} cell lines.

Spot	Fold change expressed in		AC	Protein	pI	Molecular weight (kDa)	Coverage (%)	Subcellular location	Function
	NP69-LMP1 ^{WT}	NP69-LMP1 ^{Δ232-351}							
1	↑6.43		P05388	Ribosomal protein P0	5.42	34.424	50	Cytoplasm	Transcription and translation
2	↑3.25		P21964	Catechol O-methyltransferase	5.26	30.474	52	Cytoplasm	Metabolic enzymes
3	↓4.32		P36551	Coproporphyrinogen III oxidase	8.59	50.941	28	Mitochondrion	Metabolic enzymes
4	↓2.98		P07355	Annexin A2	8.53	40.671	47	Cell membrane	Signal transduction
5	↑7.75		Q99729	Heterogeneous nuclear ribonucleoprotein A/B	6.49	36.059	37	Nucleus	Transcription and translation
6	↓4.68		P60174	Triosephosphate isomerase I	6.45	26.91	80	Cytoplasm	Metabolic enzymes
7	↑3.12		P55795	Heterogeneous nuclear ribonucleoprotein A/B	5.89	49.517	39	Nucleus	Transcription and translation
8	↑2.47		P01892	MHC class I histocompatibility antigen HLA-A alpha chain	6.32	41.171	47	Cell membrane	Immunoregulation
9	↓3.26		P22695	Cytochrome b-c1 complex subunit 2, mitochondrial	8.74	48.584	22	Mitochondrion	Electron and energy transport
10	↓2.28		P14625	Tumor rejection antigen (gp96) I	4.77	92.567	41	Endoplasmic reticulum	Molecular chaperone
11	↑2.04		P31930	Cytochrome c reductase core protein I	5.94	53.297	50	Mitochondrion	Electron and energy transport
12	↓3.45		P31943	Heterogeneous nuclear ribonucleoprotein H	5.79	49.384	45	Nucleus	Transcription and translation
13	↓3.10		Q9NR45	N-acetylneuraminic acid phosphate synthase	6.29	40.738	45	Cytoplasm	Metabolic enzymes
14	↑3.28		P50213	Isocitrate dehydrogenase 3 (NAD+)	6.47	40.022	26	Mitochondrion	Metabolic enzymes
15	↑2.43		P13674	Prolyl 4-hydroxylase	5.7	61.157	44	Endoplasmic reticulum	Metabolic enzymes
16	↓2.68		P63244	Guanine nucleotide binding protein (G protein)	7.6	35.511	65	Cytoplasm	Signal transduction
17	↓3.02		P23528	Cofilin 1	8.22	18.719	56	Nucleus and cytoplasm	Constitutive protein

AC, UniProt accession number; ↑, spot significantly upregulated in NP69-LMP1^{WT} compared with NP69-LMP1^{Δ232-351}; ↓, spot significantly downregulated in NP69-LMP1^{WT} compared with NP69-LMP1^{Δ232-351}. LMP1, latent membrane protein 1; NP69-LMP1^{Δ232-351}, mutant type LMP1; WT, wild type; pI, isoelectric point. Subcellular location and function information is derived from the NCBI database.

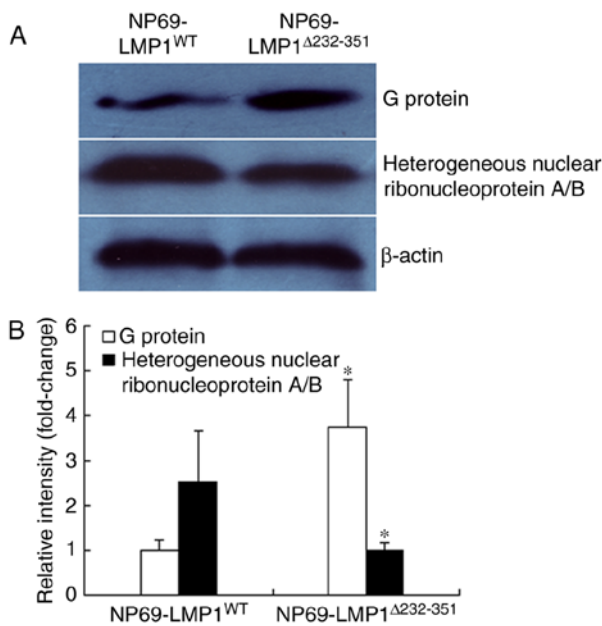


Figure 4. Expression of G protein and heterogeneous nuclear ribonucleoprotein A/B detection via western blotting. (A) Compared with NP69-LMP1^{Δ232-351} cells, the expression levels of G protein were downregulated but the expression levels of heterogeneous nuclear ribonucleoprotein A/B were upregulated in NP69-LMP1^{WT} cells. (B) Compared with NP69-LMP1^{WT} cells, the expression levels of G protein were upregulated in NP69-LMP1^{Δ232-351}. Densitometry analysis indicated that the expression of G protein and heterogeneous nuclear ribonucleoprotein A/B was significantly differential between NP69-LMP1^{Δ232-351} and NP69-LMP1^{WT} (n=3, *P<0.05 vs. NP69-LMP1^{WT}). LMP1, latent membrane protein 1; WT, wild type; LMP1^{Δ232-351}, mutant type LMP1.

NP69-LMP1^{WT}-transduced cells, the mRNA expression levels of ribosomal protein P0 and heterogeneous nuclear ribonucleoprotein A/B were increased by 5.31- and 6.45-fold, respectively (Table IV), compared with NP69-LMP1^{Δ232-351}. Two proteins, heterogeneous nuclear ribonucleoprotein A/B (greatest fold change increase) and G protein (2.07-fold change) were selected from the list of differentially expressed proteins for validation by western blotting, which reflected a similar pattern of expression to those observed in the IPG-2D gel analysis. Compared with NP69-LMP1^{Δ232-351}, NP69-LMP1^{WT} exhibited a significant increase in heterogeneous nuclear ribonucleoprotein A/B and decrease of G protein (n=3; P<0.05; Fig. 4). These results validated the expression pattern of proteins identified from the 2D gel analysis.

CTAR3 of LMP1 activates the JAK3 signaling pathway. To investigate the mechanism by which LMP1 may regulate the JAK3 signaling pathway, the NF- κ B, AP-1 or JAK3 luciferase reporters were co-transfected with wild type LMP1 or mutant LMP1^{Δ232-351} plasmid into 293 cells. The results demonstrated that the ability of mutant LMP1^{Δ232-351} in inducing transcriptional activity of NF- κ B or AP-1 LUC reporter plasmids was similar to wild type LMP1 (Fig. 5A and B). Additionally, the transcriptional activity of the JAK3 promoter was upregulated by wild type LMP1, and the extent of upregulation was associated with the concentration of wild type LMP1; however, mutant LMP1^{Δ232-351} was almost defective in activation of JAK3 reporter transcription (Fig. 5C). The results of the present study suggested that LMP1 may participate in the activation of

Table IV. Relative mRNA expression level in cancer-associated proteins differentially expressed between NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351} cell lines.

Gene	Fold change ^a expressed in NP69-LMP1 ^{WT}
Ribosomal protein P0	5.31±0.23 fold increase
Annexin A2	2.30±1.20 fold decrease
Heterogeneous nuclear ribonucleo protein A/B	6.45±0.49 fold increase
Isocitrate dehydrogenase 3	2.21±0.13 fold increase
G protein	2.07±0.47 fold decrease

^aFold change in NP69-LMP1^{WT} vs. NP69-LMP1^{Δ232-351}. LMP1, latent membrane protein 1; WT, wild type; NP69-LMP1^{Δ232-351}, mutant type LMP1.

the JAK3 signaling pathway, associated with JAK3 promoter activation via LMP1. Therefore, the results indicated that the CTAR3 of LMP1 may be a key domain required for activating the JAK3 promoter.

Discussion

EBV is a highly prevalent γ herpes virus associated with NPC and Burkett's lymphoma, and LMP1 was known generally for critical oncogenic protein coded by EBV genome (1,9,10). The studies that verified the expression of LMP1 in the majority of NPC tissues suggested that LMP1 may be closely associated with NPC genesis and invasion; however, further investigation is required (11,12). Tsao *et al* and Lo *et al* established the NP69 normal immortalization nasopharyngeal epithelium cell line *in vitro*, and reported that the cell generated a serial malignant phenotype when a LMP1 eukaryotic expression vector was introduced into the NP69 cell line (13,14). Gires *et al* (7) first reported the CTAR3 of LMP1 and confirmed the region was associated with the JAK3/signal transducer and activator of transcription (STAT) signaling pathway; however, its function in epithelial cells requires further analysis. To further investigate the functional activity of LMP1-CTAR3, a retrovirus was used to establish an NP69 cell line with stable expression of mutant LMP1^{Δ232-351} and wild type LMP1^{WT}, respectively named NP69-LMP1^{Δ232-351} and NP69-LMP1^{WT} cells in the present study. Subsequently, the biological properties of transfected NP69 cells were observed. Collectively, the results of the present study supported the findings of Tsao *et al* (13), which demonstrated that LMP1 promoted NP69 cell proliferation and transformation, increased cell growth velocity and increased multiple clone formation.

Previously, numerous studies reported the role of LMP1 transforming animal, human fibroblasts and some immortalization epithelial cells (14-16). In the present study, the results further supported the hypothesis that LMP1 may be associated with several malignancies of epithelium origin, such as NPC. In the current study, the ability of mutant LMP1^{Δ232-351} to promote proliferation was notably reduced compared with LMP1^{WT}. These results suggested that CTAR3 may participate

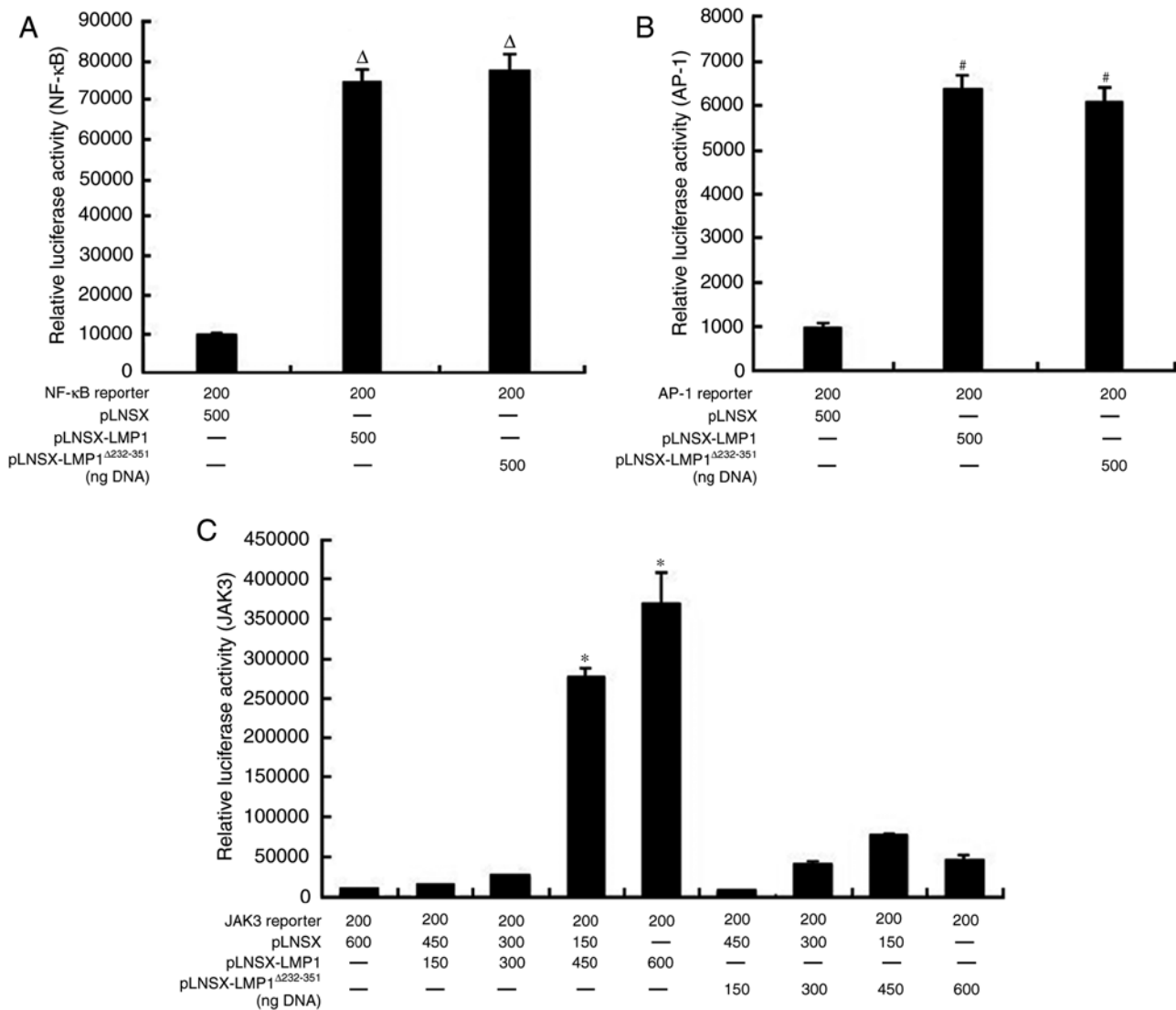


Figure 5. Ability of LMP1 protein to activate transcriptional activity of NF-κB, AP-1 and JAK3 promoters (A) Mutant LMP1^{Δ232-351} and LMP1^{WT}-induced expression of the NF-κB reporter in 293 cells (n=3, ^ΔP<0.05 vs. pLNSX). (B) Mutant LMP1^{Δ232-351} and LMP1^{WT}-induced expression of the AP-1 reporter in 293 cells (n=3, [#]P<0.05 vs. pLNSX). (C) Mutant LMP1^{Δ232-351} did not significantly induce the transcription of the JAK3 reporter within 293 cells, compared with LMP1^{WT} (n=3, ^{*}P<0.05 vs. pLNSX and pLNSX-LMP1^{Δ232-351}). Values are presented as the mean ± standard deviation. NF-κB, nuclear factor-κB; LMP1, latent membrane protein 1; WT, wild type; LMP1^{Δ232-351}, mutant type LMP1; AP-1, activator protein-1; JAK3, Janus kinase 3.

in the regulation of LMP1 associated with cell proliferation; however, whether CTAR3 is involved in JAK3/STAT3 signaling pathway requires further investigation. It has been reported that the phosphorylation of JAK3 mediates the regulation of cell proliferation (17). Therefore, LMP1-CTAR3 may activate the JAK3/STAT signaling pathway in nasopharynx epithelial cells.

Studies have reported on the role of LMP1 in the promotion of cell transformation (18,19), however to elucidate the underlying mechanism will require further research. In addition, the signaling pathway of interest, in particular the function and feature of CTAR3, lacked unified recognition and conclusion. In the present study, the protein molecule network associated with wild type LMP1^{WT} and mutant LMP1^{Δ232-351} transformed NP69 cells were investigated by proteomic analysis. The results of the present study revealed that 17 proteins were variably expressed in NP69-LMP1^{Δ232-351}-transduced cells compared with NP69-LMP1^{WT}-transduced cells. These results may

provide notable information and insight for further research. Additionally, these differential proteins were associated with cellular metabolism, signal conduction, molecular chaperones, cellular structure, immunoregulation, transcription and translation, energy and electron, molecular chaperone, as reported in the present study. Furthermore, proteins were mainly located in the endochylema, cytochondriome, endoplasmic reticulum, nucleus and cell membrane. To investigate the expression levels of differential proteins, several proteins were analyzed by western blotting and RT-qPCR. The results were similar with outcomes identified by proteomics methods in the present study (Table III and IV).

G protein, namely guanosine 5'-triphosphate-binding protein, is an important protein associated with the regulation of cellular signal transduction. Zhou *et al* (20) demonstrated that G protein phosphorylation and modification accelerated cellular apoptosis. In the present study, compared with NP69-LMP1^{WT}, the expression levels of G protein were

significantly increased within NP69-LMP1^{Δ232-351} cells. Therefore, LMP1 may downregulate the expression of G proteins to induce minor activating proteins involved in apoptosis of NP69-LMP1^{WT} cells. Hence, LMP1 may mediate cell proliferation associated with the expression of G proteins.

Heterogeneous nuclear ribonucleoprotein (hnRNP) was first described as a family of proteins that bind RNA polymerase II and is transcribed to form hnRNP particles. The hnRNP A/B proteins are among the most abundant RNA-binding proteins, forming the core of the ribonucleoprotein complex that connects with nascent transcripts in eukaryotic cells. They also recruit regulatory proteins connected with pathways related to DNA and RNA metabolism (21). He *et al* (22) have shown that hnRNP A/B proteins are dysregulated in a large number of epithelial cancer cells. In the present study, compared with NP69-LMP1^{WT}, the expression levels of G protein were significantly decreased within NP69-LMP1^{Δ232-351} cells. This suggests that LMP1 may upregulate the expression of G proteins to induce activation of proteins involved in the proliferation of NP69-LMP1^{WT} cells.

Isocitrate dehydrogenase is a rate-limiting enzyme of the tricarboxylic acid cycle and a key enzyme of cell energy metabolism, cell growth and proliferation (23). In the present study, the expression of isocitrate dehydrogenase was higher in NP69-LMP1^{WT} than that in NP69-LMP1^{Δ232-351}. The cell growth and proliferation of NP69-LMP1^{WT} was faster than NP69-LMP1^{Δ232-351}. The results of the present study suggest that LMP1-CTAR3 may mediate the regulation of isocitrate dehydrogenase expression, and affect cell metabolism and synthesis, which serves an important role in promoting cell proliferation and transformation (24).

Collectively, the findings of the present study suggested that LMP1 serves an important role in the transformation and proliferation of nasopharyngeal epithelial cells. The data of differential proteins was reported and 17 differential proteins were identified between NP69-LMP1^{WT} and NP69-LMP1^{Δ232-351}-transduced cells. These differential proteins, associated with LMP1 and the domain of CTAR3, may be involved in the regulation of cell proliferation and transformation of nasopharynx epithelial cells. The findings provide novel insight for further NPC research and may be valuable for investigating the mechanism of LMP1-associated tumors. However, how LMP1 and its CTAR3 active region regulate the expression of these differential proteins, as well as the mechanisms by which these differentially expressed proteins promote epithelial cell growth, proliferation and transformation, requires further investigation.

Acknowledgements

The authors would like to thank Dr Sai Wah Tsao (University of Hong Kong, Hong Kong, SAR, China) for NP69 cell lines.

Funding

The present study was supported by the Hunan Provincial Innovation Foundation For Postgraduate (grant no. CX2016B478), National Natural Science Foundation of China (grant no. 30470668) and Health Department Scientific Research Foundation of Hunan Province (grant no. B2006-100).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZWZ and HLZ performed the majority of the experiments in this work. YHY, ZCC and YMO performed research and analyzed the data. ZWZ, HLZ and ZMH wrote the manuscript. XSH and ZMH conceived the experimental design, were responsible for financial support and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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