

Latent membrane protein 1 encoded by Epstein-Barr virus modulates directly and synchronously cyclin D1 and p16 by newly forming a c-Jun/Jun B heterodimer in nasopharyngeal carcinoma cell line

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Abstract

Recently we confirmed that latent membrane protein 1 (LMP1) encoded by Epstein-Barr virus (EBV) accelerates a newly forming active c-Jun/Jun B heterodimer, a transcription factor, but little is known about the target gene regulated by it. In this paper, results indicated that a c-Jun/Jun B heterodimer induced by LMP1 upregulated cyclin D1 promoters activity and expression, on the contrary, downregulated p16, and maladjustment of cyclin D1 and p16 expression accelerated progression of cell cycle. Firstly, we found a c-Jun/Jun B heterodimer regulated synchronously and directly cyclin D1 and p16 in the Tet-on-LMP1-HNE2 cell line, in which LMP1 expression is regulated by Tet-on system. This paper investigated in depth function of the newly forming active c-Jun/Jun B heterodimer, and built new connection between environmental pathogenic factor, signal transduction and cell cycle.

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1. Introduction

EBV is a ubiquitous human herpesvirus that infects over 90% population of the world. LMP1, which can transform rodent fibroblasts and make nude mice tumorigenic, is considered to be a major oncogenic protein encoded by EBV (Eliopoulos and Young, 1998). LMP1 is an integral membrane protein composed of a short cytoplasmic N-terminus of 24 amino acids, six transmembrane domains of 162 amino acids and a cytoplasmic C-terminus of 200 amino acids. The

carboxyl terminus comprises two functional domains: the membrane proximal c-terminal activation region-1 (CTAR1) and the membrane distal CTAR2 (residues 351–386). LMP1 can activate AP1 signal pathway through CTAR2 function region, thus causing abnormal cell proliferation and phenotypic changes (Vogt, 2001).

Recent findings show that roles of c-Jun or Jun B, important components of AP1 family, are complex, depending on the cellular context and cell type (Deng and Karin, 1993; Mathas et al., 2002; Leaner et al., 2003; Szabowski et al., 2000). c-Jun overexpression makes tumors invasive (Wisdom et al., 1999; Smith et al., 1999), on the other hand, also, triggers apoptosis (Bossy-Wetzel et al., 1997; Wang et al., 1999a). Jun B is always deemed to the inhibitory component of AP1 transcription factor (Passegue et al., 2001),

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and it can regulate erythroid and myogenic differentiation (Jacobs-Helber et al., 2002; Chalaux et al., 1998), however, some findings demonstrate that Jun B promotes carcinogenesis (Mao et al., 2003; Robinson et al., 2001).

Further study indicates that Jun B is a negative regulator of c-Jun (Szabowski et al., 2000; Chiu et al., 1989), and Jun B represses c-Jun by forming an inactive heterodimer (Deng et al., 2003). The balance of Jun B with c-Jun activity is involved in regulating the key steps in the proliferation and differentiation process (Schlingensiepen et al., 1993). However, some findings show that c-Jun and Jun B cooperatively regulate some target genes in an AP1-dependant manner, which promotes the cell proliferation and differentiation abnormally (Mathas et al., 2002; Leaner et al., 2003).

Our study demonstrated in Tet-on-LMP1-HNE2 cell line, LMP1 triggered a signaling cascade via its CTAR2 binding TRADD/TRAF2 signaling molecular, activated JNK and then upregulated AP1 activity including c-Jun and c-fos (Liao et al., 1999a,b; Deng et al., 2003; Wang et al., 2002). Using AtlasTM Apoptosis cDNA Expression Array, we found that LMP1 induced both Jun B and c-Jun in the time- and dose-dependent manner in Tet-on-LMP1 HNE2 cell line (data not shown).

More recently, we have proved that LMP1 activates c-Jun NH2-terminal kinase (JNK), regulates protein expression and post-translational modification of Jun B and c-Jun and then induces an active c-Jun/Jun B heterodimer formation (Song et al., 2004). To further study the function of the c-Jun/Jun B heterodimer, it is crucial to find target gene regulated by it.

To be important, recently, a new direct connection was found between c-Jun and cyclin D1 related to G1/S checkpoint of cell cycle. c-Jun activated cyclin D1 promoters activity by binding to two AP1-alike sites in cyclin D1 promoter region in the HeLa cell line (Herber et al., 1994). Jun B and c-Jun were a pair of antagonistic transcriptional modulator, and the ratio of Jun B/c-Jun determined cyclin D1 transcription in the early G1 phase and balance of cell cycle. Phosphorylation of Jun B and c-Jun strengthened their transcriptional activity, regulated their dynamic balance, and at last modulated cyclin D1 promoters activity, influencing cyclin D1 expression (Bakiri et al., 2000).

In the meantime, also, a new direct connection is recently found between Jun B and p16, a negative modulator related to cell cycle. Three AP1-like binding sites were identified in the mice p16 promoter region. Jun B can activate directly p16 transcription through binding to these sites (Passegue and Wagner, 2000). Using Domain Architecture Retrieval Tool (<http://www.ncbi.nlm.nih.gov/Structure/Dart/Lexington/html/overview.html>), we found that Jun B domains between human and mice are highly homologous (99%), and there is only an amino acid difference in the basic leucine zipper domain (bZIP) of Jun B. We further predicted that four putative AP1 bind sites exist in the human p16 promoters using bioinformatics (<http://www.cbrc.jp/research/db/TFSEARCH.html>).

Using AtlasTM human cDNA expression array I (Clontech) detecting normal human nasopharyngeal tissue and nasopharyngeal carcinoma, we found cyclin D1 overexpression in the nasopharyngeal carcinoma at the transcriptional level (Li et al., 1998; Xie et al., 2000). In the meantime, we proved cyclin D1 protein expression increased gradually in the multistage tissue of the simple hyperplasia/metaplasia, atypical hyperplasia/metaplasia and nasopharyngeal carcinoma during nasopharyngeal carcinogenesis, strengthening significantly especially in the atypical hyperplasia/metaplasia tissue (data not shown). Further study indicated LMP1 encoded by EBV regulated cyclin D1, thus led to disorder of proliferation and maladjustment of apoptosis (Zhao et al., 2000, 2001).

On the other hand, point mutation of p16 (Sun et al., 1995) occurs rarely in the human nasopharyngeal carcinomas, but there exists frequent inactivation of p16 (Huang et al., 2001; Baba et al., 2001), or lack (Gulley et al., 1998; Makitie et al., 2003; Lo et al., 1995), or hypermethylation of its promoters (Jefferies et al., 2001; Chang et al., 2003; Lo et al., 1996). Restoration of p16 in nasopharyngeal carcinoma inhibited significantly tumorigenic potential (Wang et al., 1999b; Lee et al., 2003). Meanwhile, EBV inhibited p16 expression in human fetal nasopharyngeal epithelial cells escaping from the replicative senescence (Yang et al., 2003).

These studies above all suggest that cyclin D1 and p16 may be adjusted synchronously by the c-Jun/Jun B heterodimer induced by LMP1.

So, based on progress on study of literature and our work done (Song et al., 2004), in this paper, we want to investigate in depth whether a c-Jun/Jun B heterodimer induced by LMP1 could regulate cyclin D1 and p16 synchronously, which are closely related to cell cycle, and accelerate cell cycle progression.

2. Materials and methods

2.1. Cell culture

HNE2 is EBV-negative, poorly differentiated NPC cell line. Tet-on-LMP1-HNE2 was constructed by transfecting LMP1 expressin plasmid and tet-on system into HNE2. In the Tet-on-LMP1-HNE2, LMP1 and tet-on was stably integrated, the expression of LMP1 will be turned on by tetracycline (Doxycycline, Dox). Two cell lines were established by Cancer Research Institute, Xiangya School of Medicine, Central South University (Liao et al., 1999a,b, 2001). Tet-on-LMP1-HNE2 cells were cultured in RPMI 1640 medium (GIBCO BRL) containing 100 µg/ml G418, 50 µg/ml hygromycin and 10% FCS (GIBCO BRL) in a humidified 5% CO₂ atmosphere at 37 °C. To induce LMP1 expression, cells were treated with Dox at 0, 0.006, 0.06, 0.6 and 6.0 µg/ml, respectively. In the meanwhile, with 0.6 µg/ml Dox induction, cell was collected at the time points of 0, 0.5, 1, 2, 4, 8, 12, 18 and 24 h.

2.2. Protein extraction

After treatment with Dox, the cells were washed with ice-cold PBS and lysed by 10 min incubation on ice with lysis buffer (50 mol/ml Tris Cl PH 7.6, 400 mol/ml NaCl, 1 mol/ml EDTA, 1 mol/ml EGTA, 1% NP40, 0.5 µg/ml leupeptin, 0.5 µg/ml aprotinin, 0.5 µg/ml pepstatin, 1 mol/ml PMSF). The lysate was centrifuged for 5 min at 14,000 rpm.

For electrophoresis mobility shift assays (EMSA) (Andrews and Faller, 1991), nuclear extracts were prepared by growing cells to confluence, washing them three times in cold phosphate-buffered saline (PBS), and harvesting them by scraping. Cells were precipitated in a microcentrifuge, rinsed with hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl], and resuspended in two pellet volumes of hypotonic buffer. After 5 min on ice, the suspension was lysed by adding 1 µl of 5% Nonidet P-40 and agitated by pipetting through a Pasteur pipette. Nuclei were then rocked for 30 min at 4 °C to ensure complete lysis with buffer C [20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA]. Dithiothreitol (DTT) (0.5 mM), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg of leupeptin/ml, 1 µg of aprotinin/ml, 1 mM sodium vanadate, and 20 mM sodium fluoride were added to each solution just prior to use. Protein concentrations were checked by BCA assay reagent (Pierce Chemical).

2.3. Western blotting

One hundred micrograms aliquots of protein lysates were mixed with the sample buffer and boiled for 5 min. Samples were then resolved on 8% polyacrylamide gel containing SDS, and transferred to nitrocellulose membrane (Schleicher & Schuell) by electro blotting. The membrane was incubated in blocking buffer (TBS containing 5% skim milk and 0.1% Tween 20) for 2 h, followed by incubation with primary antibody diluted to 1:1000. The membrane was washed in TBS containing 0.1% Tween 20. Specifically secondary antibody was detected using peroxidase-conjugated anti IgG at 1:10,000. Relative proteins were detected by supersignal chemiluminescence system (ECL, Pierce) followed by exposure to autoradiographic film. The Nitrocellulose membrane was stripped with stripping buffer and reblotted. LMP1 (cs1-4, DAKO), AP1(D)-G, widely reactive with c-Jun, Jun B and Jun D (sc-44-G, Santa Cruz), c-Jun (sc-1694, Santa Cruz), Jun B (sc-8051, Santa Cruz), a-tubulin (sc-5286, Santa Cruz), p16 (sc-759, sc-2037) and cyclin D1 (H-295, Santa Cruz).

2.4. Probes

Two double-stranded probes containing AP1 binding site (underlined) in cyclin D1 promoter region (Herber et al., 1994) were synthesized as follows: probe1: 5'-AAAAAAT-GAGTCAGAATGGAG-3' (−929 to −935); probe2: 5'-CA-ACAGTAACGTCACACGGAC-3' (−45 to −52). Four double-stranded probes containing AP1 binding site (under-

lined) in p16 promoter region (Bakiri et al., 2000; Passegue and Wagner, 2000) were predicted by bioinformatics and synthesized as follows: probe1: 5'-AAAAGTATTAGTCA-CAGGAAA-3' (−14 to −23); probe2: 5'-GTCTCATTCT-GTCACCCAGGC-3' (−530 to −539); probe3: 5'-CTTCC-AATGACTCCCTCCCCA-3' (−1162 to −1171); probe4: 5'-GGGTTTCTGACTTAGTGAACC-3' (−1380 to −1387). Universal AP1 Probe (Lee et al., 1987; Briggs et al., 1986) for positive control and SP1 probe (Briggs et al., 1986; Vallone et al., 1997) for intrinsic control were also synthesized as follows: AP1: 5'-TTCCGGCTGACTCATCAAGCG-3'; SP1: 5'-ATTCGATCGGGGCGGGGCGAGC-3. All probes as above were synthesized and labeled with biotin at 3'-terminal by Laboratory of Molecular Technology, SAIC-Frederick National Cancer Institute, USA.

2.5. Transfection conditions and luciferase assay

Plasmids purification were followed as instruction of QIAGEN Plasmid Maxi Kit (Qiagen). Before transfection, cells were seeded in six-well plates overnight. Transfection was performed using SuperFect transfection Reagent kit (Qiagen). The luciferase activity was detected with luciferase assay kit (Promega). The plasmids were used as follows: p16 promoters reporter plasmid was kindly presented by Dr. Peters G (Hara et al., 1996), cyclin D1 promoters luciferase reporter plasmid was kindly provided by Prof. Strauss (Hinz et al., 1999) and β-galactosidase expression plasmid (pRSVβ-gal) was gently provided by Dr. Perkins ND (Chapman and Perkins, 2000).

2.6. Electrophoresis mobility shift assay (EMSA) and Super-EMSA

For EMSA (LightShift™ Chemiluminescent EMSA Kit Pierce), 5 µg of nuclear extract was incubated with 20 fmol of the end-labeled with biotin double-stranded oligonucleotide probes in reaction buffer for 20 min at room temperature. For supershift reactions, the optimal concentration of each antibody was determined by titration into the EMSA reaction mixture. All antibodies were added at the same concentration (12.5 ng/µl) to correspond to the optimal concentration of the least potent antibody that showed a discernible supershift. Samples were resolved on a nondenaturing 4% polyacrylamide 2% glycerol gel, transferred to Bodine® B Nylon membrane, avidin-HRP to probes and visualized and quantitated with a PhosphorImager. Transcription factor SP1 bindings sequence was used as a control probe. The supershift antibodies were used as follows: AP1(D)-G, widely reactive with c-Jun, Jun B and Jun D (sc-44-G, Santa Cruz), c-Jun (sc-1694, Santa Cruz), Jun B (sc-8051, Santa Cruz).

2.7. DNA content assay

Untreated and treated cells were collected, after cultured in the presence or absence of Dox for the indicated time,

rinsed with PBS and suspended in 75% ethanol at -20°C overnight. Fixed cells were centrifuged and washed with PBS twice. For detecting DNA content, cells were soaked in the dark in 400 μl PBS containing 50 $\mu\text{g}/\text{ml}$ of PI and 0.1% of RNase A at 25°C for 30 min. Stained cells were analyzed by flow cytometry (model: ELITE, laser wavelength: 488 nm, power: 15 mW, Beckman–Coulter, USA). The percentage of cell in G1, S and G2 phases was determined using the MULTICYCLE software program (PHEONIX).

2.8. Immunocytometry

Cells were washed with PBS and resuspended in 75% ethanol at -20°C overnight. Fixed cells were centrifuged, washed with PBS and treated with 0.25% Triton X-100 in PBS for 5 min. After the addition of PBS and centrifugation, the samples were incubated with mouse mAb to human cyclin D1 (DCS-6 DAKO) or to human p16 (sc-1661, Santa Cruz) at -4°C overnight. The antibody was diluted in PBS containing 1% BSA and applied at a ratio of 0.25 μg of mAb/ 5×10^5 cells. After washed with 1% BSA, the cells were incubated in the dark with FITC-conjugated sheep anti-mouse IgG (F2266, Sigma) for 30 min. After washed with 1% BSA again, the cells were stained with 50 $\mu\text{g}/\text{ml}$ PI and 0.1% RNase in 400 μl PBS for 30 min. Cells were analyzed for DNA content and FITC positivity on a FACSsort flow cytometry and Cellquest software (Becton Dickinson).

3. Results

3.1. Binding activity of the c-Jun/Jun B heterodimer with cyclin D1 and p16 promoters induced by LMP1

It was found that LMP1 expression by western blot in the Tet-on-LMP1-HNE2 cells treated with Dox at 0, 0.006, 0.06, 0.6, 6.0 $\mu\text{g}/\text{ml}$, respectively, increased in a dose-dependent manner, reached to peak at 0.6 $\mu\text{g}/\text{ml}$ (Fig. 1A). Thus, cells were treated with Dox at 0.6 $\mu\text{g}/\text{ml}$, and nuclear protein was extracted at the time points of 0, 0.5, 1, 2, 4, 8, 12, 18 and 24 h. LMP1 expression was found to increase in a time-dependent manner (Fig. 1B).

Recent study has proved in the Tet-on-LMP1-HNE2 cells treated with Dox for 12 h at 0.6 $\mu\text{g}/\text{ml}$, LMP1 can accelerate effectively c-Jun/Jun B heterodimers formation, and strengthen significantly AP1 DNA-binding activity of the heterodimers (Song et al., 2004).

Therefore, on the condition, Tet-on-LMP1-HNE2 cells were treated with 0.6 $\mu\text{g}/\text{ml}$ Dox for 12 h, and nuclear protein was extracted. It was found by Super-EMSA two subunits of c-Jun and Jun B could both bind to cyclin D1 promoters region (sequences of binding sites: 5'-TGAGTCA-3') (Fig. 2).

In the meanwhile, we predicted that four putative AP1-binding sites exist in the human p16 promoters region by bioinformatics (<http://www.cbrc.jp/research/db/>

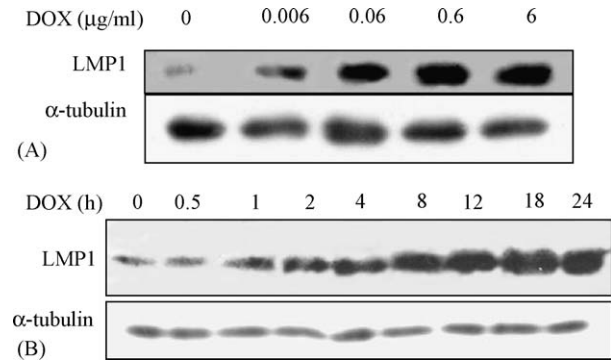


Fig. 1. (A) Dose response of Dox induction of LMP1 expression. Tet-on-LMP1-HNE2 cells were treated with Dox at 0.006, 0.06, 0.6 and 6 $\mu\text{g}/\text{ml}$, respectively, as a control without Dox induction, and the whole protein was extracted. 100 μg of cellular protein was subjected to electrophoresis on 8% SDS-polyacrylamide gel and Western blot analysis. LMP1 (~ 60 kDa) is identified by probing with a specific antibody described in Section 2. Signals were normalized using expression of α -tubulin as control. (B) Time course of Dox induction of LMP1 expression. Tet-on-LMP1-HNE2 cells were treated with Dox at 0.6 $\mu\text{g}/\text{ml}$. The whole protein was extracted, respectively, at the time points of 0, 0.5, 1, 2, 4, 8, 12, 18 and 24 h; 100 μg of cellular protein was subjected to electrophoresis on 8% SDS-polyacrylamide gel and Western blot analysis. LMP1 (~ 60 kDa) is identified by probing with a specific antibody described in Section 2. Signals were normalized using expression of α -tubulin as control.

TFSEARCH.html). To detect whether the c-Jun/Jun B heterodimer combined with p16 promoter region, by Super-EMSA, results indicated two subunits of c-Jun and Jun B could both bind to p16 promoter region (sequences of binding sites: 5'-TTCTGTCACC-3') (Fig. 3).

3.2. c-Jun/Jun B heterodimer modulated cyclin D1 and p16 promoters activity as well as their expression with induction of LMP1

Cyclin D1 luciferase reporter plasmid and β -gal expression plasmid were cotransfected into HNE2 cells and Tet-

Fig. 2. c-Jun/Jun B heterodimer DNA-binding activity in the cyclin D1 promoter region in the Tet-on-LMP1-HNE2 cells treated with 0.6 $\mu\text{g}/\text{ml}$ of Dox for 12 h. Nuclear extracts were prepared from cells. Gel supershift assays were performed by initially incubating 5 μg of nuclear protein with 20 fmol of 3' biotin-labelled duplex containing a single AP1 binding site for 20 min at room temperature. Various antibodies (1 μg) described in Section 2 against either c-Jun or Jun B were then added to the binding reaction mixtures and the reaction was allowed to proceed for an additional 30 min at room temperature. Universal AP1(D)-G antibody for a positive control is widely reactive with c-Jun, Jun B and Jun D (sc-44-G, Santa Cruz). Universal AP1 Probe (Lee et al., 1987; Briggs et al., 1986) is used as a positive control. The specific supershifted AP1 components are indicated by arrows. Signals were normalized using binding activity of SP1 as intrinsic control. Two experiments were performed that showed similar results. (A) c-Jun subunit DNA-binding activity with synthesized probes as described above of the c-Jun/Jun B heterodimer in the cyclin D1 promoter region in the Tet-on-LMP1-HNE2 cells treated with 0.6 $\mu\text{g}/\text{ml}$ of Dox for 12 h. (B) Jun B subunit DNA-binding activity with synthesized probes as described above of the c-Jun/Jun B heterodimer in the cyclin D1 promoter region in the Tet-on-LMP1-HNE2 cells treated with 0.6 $\mu\text{g}/\text{ml}$ of Dox for 12 h.

on-LMP1-HNE2 cells treated with Dox for 24 h at 0, 0.006, 0.06, 0.6, 6 $\mu\text{g/ml}$, respectively. It was found that cyclin D1 luciferase activity increase gradually, compared to 0 $\mu\text{g/ml}$, up to over six times at 6 $\mu\text{g/ml}$ (* $P < 0.05$) (Fig. 4A).

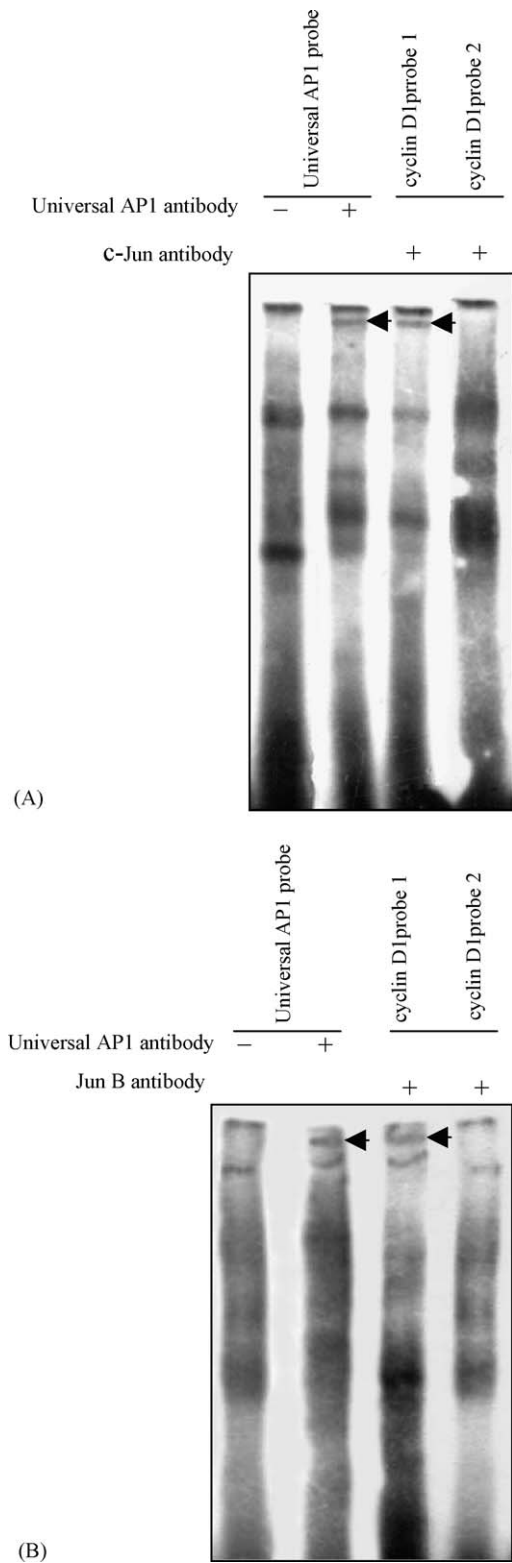


Fig. 2.

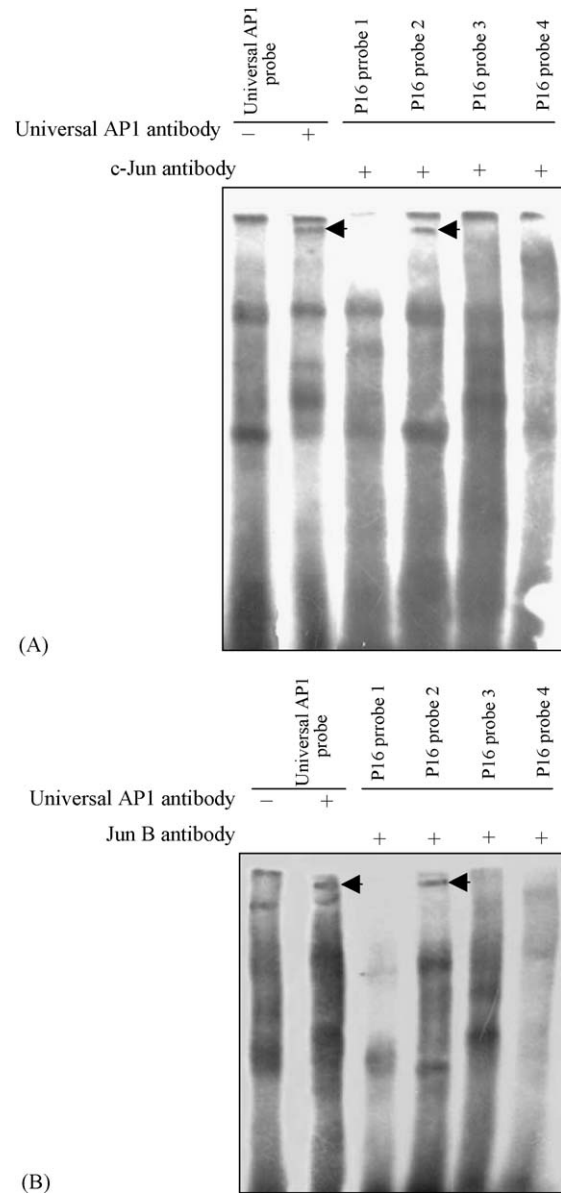


Fig. 3. c-Jun/Jun B heterodimer DNA-binding activity in the p16 promoter region in the Tet-on-LMP1-HNE2 cells treated with 0.6 $\mu\text{g/ml}$ of Dox for 12 h. Nuclear extracts were prepared from cells. Gel supershift assays were performed by initially incubating 5 μg of nuclear protein with 20 fmol of 3'-biotin-labelled duplex containing a single AP1 binding site for 20 min at room temperature. Various antibodies (1 μg) described in Section 2 against either c-Jun or Jun B were then added to the binding reaction mixtures and the reaction was allowed to proceed for an additional 30 min at room temperature. Universal AP1(D)-G antibody for a positive control is widely reactive with c-Jun, Jun B and Jun D (sc-44-G, Santa Cruz). Universal AP1 Probe (Lee et al., 1987; Briggs et al., 1986) is used as a positive control. The specific supershifted AP1 components are indicated by arrows. Signals were normalized using binding activity of SP1 as intrinsic control. Two experiments were performed that showed similar results. (A) c-Jun subunit DNA-binding activity with synthesized probes as described above of the c-Jun/Jun B heterodimer in the p16 promoter region in the Tet-on-LMP1-HNE2 cells with 0.6 $\mu\text{g/ml}$ of Dox for 12 h (B) Jun B subunit DNA-binding activity with synthesized probes as described above of the c-Jun/Jun B heterodimer in the p16 promoter region in the Tet-on-LMP1-HNE2 cells with 0.6 $\mu\text{g/ml}$ of Dox for 12 h.

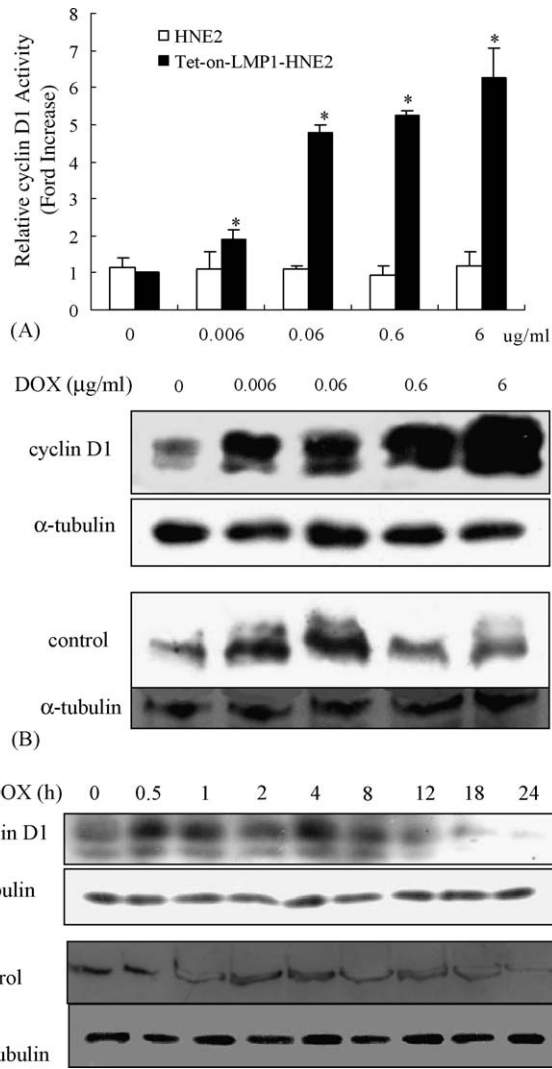


Fig. 4. (A) Dose response of cyclin D1 luciferase activity in Tet-on-LMP1-HNE2 cells treated, respectively, with 0, 0.006, 0.06, 0.6, 6 μg/ml of Dox, as a control in HNE2 cells. Cyclin D1 luciferase reporter plasmid and β-gal expression plasmid were cotransfected into HNE2 cells and Tet-on-LMP1-HNE2 cells treated with Dox for 24 h at 0, 0.006, 0.06, 0.6, 6 μg/ml, respectively. Cells were extracted in 100 μl of lysis buffer, and cyclin D1 activity was measured as described in Section 2. The level of cyclin D1 activity increased evidently compared to that of without Dox induction ($*P < 0.05$, compared to 0 μg/ml). Results are means of at least three experiments, and Error bars indicate 1 standard deviation from the mean. The expression plasmid of β-gal (pRSVβ-gal) as an intrinsic control. (B) Dose response of Dox induction of cyclin D1 expression. Tet-on-LMP1-HNE2 cells were treated with 0, 0.006, 0.06, 0.6, 6 μg/ml of Dox, as a control without Dox induction at every dose. Cellular protein (100 μg) was subjected to electrophoresis on 8% SDS-polyacrylamide gel and Western blot analysis. Cyclin D1 is identified by probing with a specific antibody described in Section 2. Signals were normalized using expression of α-tubulin as control. (C) Time course of cyclin D1 protein expression in the Tet-on-LMP1-HNE2 cells treated with with 0.6 μg/ml of Dox, as a control without Dox induction at every time point. Cells were treated with 0.6 μg/ml of Dox. 100 μg of cellular protein was subjected to electrophoresis on 10% SDS-polyacrylamide gel and Western blot analysis. Cyclin D1 is identified by probing with a specific antibody described in Section 2. Signals were normalized using expression of α-tubulin as control.

Based on the c-Jun/Jun B heterodimer binding with cyclin D1 promoters and regulating its activity with the induction of LMP1, cyclin D1 protein expression was further studied. The results demonstrated it increased in a dose-dependent manner (Fig. 4B).

Our recent study has confirmed LMP1 protein expression in the Tet-on-LMP1-HNE2 cells induced by Dox at 0.6 μg/ml

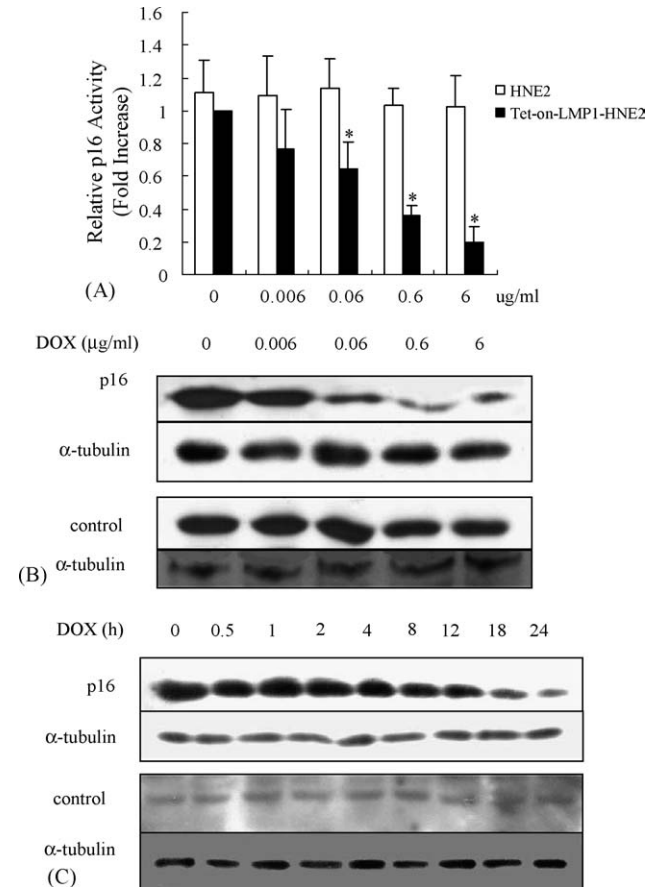


Fig. 5. (A) Dose response of p16 luciferase activity in Tet-on-LMP1-HNE2 Cells were treated, respectively, with 0, 0.006, 0.06, 0.6, 6 μg/ml of Dox, as a control in HNE2 Cells. p16 luciferase reporter plasmid and β-gal expression plasmid were cotransfected into HNE2 cells and Tet-on-LMP1-HNE2 cells treated with Dox for 24 h at 0, 0.006, 0.06, 0.6, 6 μg/ml, respectively. Cells were extracted in 100 μl of lysis buffer, and p16 activity was measured as described in Section 2. The level of p16 activity decreased evidently compared to that of no induction ($*P < 0.05$, compared to 0 μg/ml). Results are means of at least three experiments, and error bars indicate 1 standard deviation from the mean. The expression plasmid of β-gal (pRSVβ-gal) as an intrinsic control. (B) Dose response of Dox on the induction of p16 expression. Tet-on-LMP1-HNE2 cells were treated with 0, 0.006, 0.06, 0.6, 6 μg/ml of Dox, as a control without Dox induction at every dose. Cellular protein (100 μg) was subjected to electrophoresis on 15% SDS-polyacrylamide gel and Western blot analysis. P16 is identified by probing with a specific antibody described in Section 2. Signals were normalized using expression of α-tubulin as control. (C) Time course of p16 expression with induction of 0.6 μg/ml of Dox. Tet-on-LMP1-HNE2 cells were treated with 0.6 μg/ml of Dox, as a control without Dox induction at every time point. Cellular protein (100 μg) was subjected to electrophoresis on 15% SDS-polyacrylamide gel and Western blot analysis. p16 is identified by probing with a specific antibody described in Section 2. Signals were normalized using expression of α-tubulin as control.

Table 1
Cell cycle distribution (%) of Tet-on-LMP1-HNE2 cells treated with 0.6 µg/ml Dox at the different time points

Phases	Time points (h)								
	0	0.5	1	2	4	8	12	18	24
Dox(-)/G1	55.8	53.9	53.1	49.2	41.7	48	57.8	65.7	66.3
Dox(+)/G1	55.8	57.7	55.1	52.5	42.4	52.2	58	70.2	74.1
Dox(-)/S	40.8	43.2	43.7	48.9	52.1	40.6	29.5	24.1	26.4
Dox(+)/S	40.8	40.2	42.6	46	51.8	36.5	28.6	20.9	21.7
Dox(-)/G2	3.4	2.9	3.2	1.9	8.7	11.4	14.5	10.2	7.3
Dox(+)/G2	3.4	2.1	2.3	1.5	5.8	11.3	13.4	8.9	4.2

increased in a time-dependent manner, while a c-Jun/Jun B heterodimer increased gradually, then decreased after 18 h (Song et al., 2004). To further confirm the c-Jun/Jun B heterodimer accelerated by LMP1 regulated cyclin D1 protein expression, Western blot analyses were performed at the different time points. Results demonstrated that cyclin D1 expression increased from 0.5 h, maintaining at higher level up to 8 h, decreasing from 12 h and extremely weak at 24 h (Fig. 4C).

In the same condition, also, data showed that p16 promoters activity and its protein expression decreased gradually (Fig. 5).

3.3. Effects of LMP1 encoded by EBV on cell cycle by inducing a c-Jun/Jun B heterodimer modulating cyclin D1 and p16 expression directly and synchronously

Cyclin D1 and p16 is, respectively, regarded as key positive and negative modulators of network of cell cycle (Sarasin, 2003). Based on LMP1 inducing a c-Jun/Jun B heterodimer formation (Song et al., 2004), and then upregulating cyclin D1 (Fig. 4), while downregulating p16 promoters activity and expression (Fig. 5), we further explored the effects of the cyclin D1 and p16 cooperatively on cell cycle at the different time points.

As a control without Dox induction at every time point, DNA content of Tet-on-LMP1-HNE2 cells treated with 0.6 µg/ml Dox was detected in the indicated time points by flow cytometry for determining changes of cell cycle. Data showed that after Dox induction the percentage of cells at

almost every time point in the G1 phase ascended at some degree, and meanwhile descending in S and G2 phases (Table 1, Fig. 6A). This suggested the cells traverse through the cell cycle more quickly in the presence of LMP1 with the induction of Dox.

To further elucidate the relativity between cell percentage and cyclin D1 and p16 expression in the time-course, the mean fluorescence intensity of cyclin D1 and p16 was detected by immunocytometry. The results indicated cyclin D1 strengthened significantly only in 0.5 h, kept in a high state from 0.5 to 8 h, and decreased in evidence from 8 to 24 h (Table 2, Fig. 6B), while p16 weakened gradually (Table 3, Fig. 6C). Thus, these results indicates that the mean fluorescence intensity of cyclin D1 and p16 matches much ones of cyclin D1 and p16 at protein level (Figs. 4C and 5C).

4. Discussion

Our late study confirmed that LMP1 induced formation of active a c-Jun/Jun B heterodimer in Tet-on-LMP1-HNE2 cell line, and the heterodimer could bind to the AP1-DNA-binding motif (Song et al., 2004).

In this paper, we further found that LMP1 accelerated a c-Jun/Jun B heterodimer binding, respectively, with the site (5'-AAAAAATGAGTCAGAATGGAG-3', -929 to -935) in cyclin D1 promoter region (Fig. 2) and the site (5'-GTCTCATTCTGTCACCCAGGC-3', -530 to -539) in p16 promoter region (Fig. 3), upregulating cyclin D1 (Fig. 4) while downregulating p16 promoters activity and protein ex-

Table 2
Cyclin D1 mean fluorescence intensity in the Tet-on-LMP1-HNE2 cells treated with 0.6 µg/ml of Dox at the different time points

	Time points (h)								
	0	0.5	1	2	4	8	12	18	24
Dox(-)	356.34	385.21	381.04	375.85	376.00	377.83	367.97	350.52	340.62
Dox(+)	356.34	370.45	370.99	369.78	370.55	365.89	359.12	348.64	338.68

Table 3
p16 mean fluorescence intensity in the Tet-on-LMP1-HNE2 cells treated with 0.6 µg/ml of Dox at the different time points

	Time points (h)								
	0	0.5	1	2	4	8	12	18	24
Dox(-)	656.13	635.79	630.63	601.88	569.74	516.72	484.56	473.45	468.72
Dox(+)	656.13	621.89	614.76	590.98	555.76	510.57	475.67	461.67	455.89

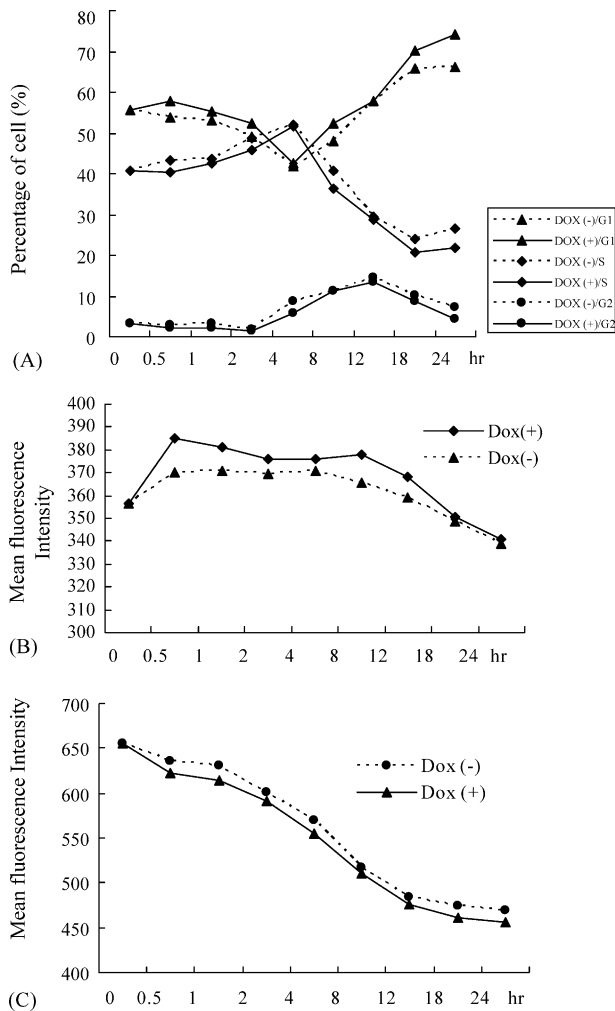


Fig. 6. (A) Distribution (%) of the Tet-on-LMP1-HNE2 cells in the phases of cell cycle treated with Dox at the different time points, as a control without Dox induction at every time point. Cells were treated with 0.6 $\mu\text{g}/\text{ml}$ Dox for different time as indicated above. 2×10^6 cells were fixed with ethanol, stained with propidium iodide, and analyzed with Becton Dickinson flow cytometer. Dead cells were excluded by propidium iodide staining. The data represent one of three independent experiments performed with consistent results. In order to give visually the changes (A) was constructed according to the results in Table 1. (B) The mean fluorescence intensity of cyclin D1 in the all phases with Dox induction at the different time points in Tet-on-LMP1-HNE2 cells, as a control without Dox induction at every time point. Cells were treated with 0.6 $\mu\text{g}/\text{ml}$ Dox for different time as indicated above. 2×10^6 cells were fixed with paraformaldehyde and ethanol, stained with propidium iodide. Cyclin D1 expression was analyzed by immunostaining with anti-cyclin D1 antibody followed by FITC-conjugated anti-mouse secondary antibody by Becton Dickinson flow cytometry. Dead cells were excluded by propidium iodide staining. Each staining was performed at least three times with similar results. (B) was constructed according to the results in Table 2. (C) The mean fluorescence intensity of p16 with Dox induction at the different time points in Tet-on-LMP1-HNE2 cells, as a control without Dox induction at every time point. Cells were treated with 0.6 $\mu\text{g}/\text{ml}$ Dox for different time as indicated above. 2×10^6 cells were fixed with paraformaldehyde and ethanol, stained with propidium iodide. p16 expression was analyzed by immunostaining with anti-p16 antibody followed by FITC-conjugated anti-mouse secondary antibody by Becton Dickinson flow cytometry. Dead cells were excluded by propidium iodide staining. Each staining was performed at least three times with similar results. (C) was constructed according to the results in Table 3.

pression (Fig. 5). To be interesting, reasons why cyclin D1 and p16 expression induced by LMP1 shows opposite behavior may lie in the changes of amount of a c-Jun/Jun B heterodimer binding to between cyclin D1 and p16 promoter region, or an increasing c-Jun/Jun B heterodimer binding partly to enhancer of cyclin D1, while partly to silencer of p16. Also, many data shows NF- κB regulates cyclin D1 expression and G0/G1-to-S-phase transition of cell cycle by binding three sites in cyclin D1 promoter region (Hinz et al., 1999; Guttridge et al., 1999). In the meantime, we also found crosstalk between AP-1(c-Jun) and NF- κB (p50 and p65) signal pathways (Yang et al., 2003; Delerive et al., 1999; Yin et al., 2001; Liao et al., 1999a,b; Wang et al., 2003). Thus, in Tet-on-LMP1-HNE2 cell line, LMP1 may mediate NF- κB and AP1 signal pathways, and integrate signals on cyclin D1 (Deng et al., 2003; Wang et al., 2002), which is involved in the imbalance of cell proliferation and apoptosis (Zhao et al., 2000, 2001). Taken together, in some degrees, our data confirmed LMP1 modulated cyclin D1 and p16 synchronously via active a c-Jun/Jun B heterodimer formation.

Between phases of cell cycle, there exist a series of G1/S, G2/M and, etc. checkpoints, which supervise and modulate phases of cell cycle to make them normal transition, thus guarantee cell cycle progress orderly. Especially, G1/S checkpoint is vital (Molinari, 2000).

Tumors as cell cycle diseases, G1/S checkpoint is often of maladjustment during multistage carcinogenesis, even in the precancerous phase. This may lie mainly in both positive regulator cyclin D1 and negative regulator p16, which are closely correlated with the regulation of G1/S checkpoint (Lukas et al., 1995).

In the physiological state, cyclin D1 expression of cells in the logarithmic growth phase peaks in G0 phase with the stimulus to mitotic signal. Cyclin D1 appears in G0 and early G1 phases, degrades before cells into S phase and disappears in S and G2/M phases in the most conditions. Therefore, cyclin D1 is considered as an important marker of G0–G1 transition (Tam et al., 1994).

Thus, cyclin D1 is firstly expressed when cell is stimulated into cell cycle, and cyclin D1 becomes vital tache between extracellular growth factor, signal transduction and cell cycle (Arber et al., 1996). Cyclin D1 combines with cyclin-dependent-kinase 4/6 (CDK4/6) into complex and makes it active. Then Rb is phosphorylated by active CDK4/6 and releases E2F, which binds to Rb. E2F enters into cell nucleus and triggers the expression of downstream target genes related to cell cycle, which promote cells overpassing G1/S checkpoint (Sherr, 1995, 1996; Donjerkovic and Scott, 2000; Bartek et al., 1996). Furthermore, once cells in the cell cycle overpass G1/S checkpoint, there existing in the irreversible state (Bartek et al., 1999). On the contrary, indolocarbazoles, a selective inhibitor of cyclin D1/CDK4, could make cells of tumor stop growing, blocking in G1 phase (Petty et al., 2003; Zhu et al., 2003; Engler et al., 2003). Recent study demonstrated transient cyclin D1 expression was sufficient for accelerating normal reproduction and growth of cells (Nelsen

et al., 2001), but overexpression triggered cell cycle drive engine strengthening uninterrupted, disorder of G1/S checkpoint to make unrepaired cell into next phase, thus leading to genome more unstable, and quickening up tumor malignant progression (Robles et al., 1996; Zhou and Elledge, 2000).

But, p16 is regarded as an inhibitor of CDK and negative regulator of cell cycle G1/S checkpoint, the amino-terminus of p16 is of the same homogenous domain as of cyclin D1. p16 competes binding sites to CDK with cyclin D1. Obviously, p16 may prevent G1/S checkpoint from dysfunction, even carcinogenesis, and counteract CDK overactivation by cyclin D1 (Sarasin, 2003).

Recently, data demonstrated that p16 could lead cells to arrest in G0/G1 phase as a inhibitor of CDK in the nasopharyngeal carcinoma (Chow et al., 2000). Lack of p16 made cell loss of negative control on CDK, resulting in more cells across G1/S checkpoint, and accelerating cell overgrowth (Shibosawa et al., 2000).

Also, more data demonstrated that p16 cooperation with cyclin D1 were critical for growth arrest during mammary involution (Gadd et al., 2001). The imbalance between p16 and cyclin D1 expression was closely correlated to tumor progression in bladder cancer (Yang et al., 2002), both inactivation of p16 and overexpression of cyclin D1 was much related to upper aerodigestive tract, primary oral cancer and neuroendocrine tumors of the lung (Papadimitrakopoulou et al., 2001; Sartor et al., 2001; Beasley et al., 2003). Apparently, many types of tumors displayed the abnormal expression of p16 and cyclin D1. The imbalance between cyclin D1, activator of CDK and p16, inhibitor of CDK, strengthened the drive of cell cycle and weakened the surveillance of cell cycle. Therefore, the imbalanced may trigger more cells injured without being restored into next cell cycle, and lead cell proliferation abnormally, strengthening genome instability and accelerating process of carcinogenesis (Molinari, 2000; Zhou and Elledge, 2000; Bindels et al., 2002; Izzo et al., 1998).

Our data suggested more G0 (resting phase) cells reentered into the G1 phase of cell cycle, while S and G2 (fission phase) cells traverse through the cell cycle more quickly and enter next cell cycle with increasing gradually cyclin D1 mean fluorescence intensity and decreasing p16 (Fig. 6B and C). Thus, this may make G1/S checkpoint maladjusted, trigger cells abnormal multiplication and speed further up tumorous malignant progression. This paper establishes direct connection between LMP1 of environmental pathogenic factor, AP1 signal transduction pathway and cell cycle. Also, it offers theory gist on cell-cycle-related drug design and target gene therapy (Petty et al., 2003; Viallard et al., 2001).

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