

LMP1 promotes nasal NK/T-cell lymphoma cell function by eIF4E via NF- κ B pathway

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Abstract. Nasal natural killer T-cell lymphoma (NKTL) is a highly malignant tumor that is closely associated with Epstein-Barr virus (EBV) infection. Latent membrane protein 1 (LMP1) is encoded by EBV and plays an important role in EBV-induced cell transformation. Therefore, we assessed the function of LMP1 as a stimulant of NKTL progression and the underlying mechanism. A human EBV-positive NKTL cell line (SNK-6) was transfected with pcDNA3.1-LMP1, LV-LMP1 shRNA or LV-eukaryotic translation initiation factor 4E (eIF4E)-shRNA. Then, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to assess the proliferation of SNK-6 cells, and cell migration and invasion were analyzed by Transwell chamber assay. Flow cytometry was used to analyze the cell cycle and apoptosis. The results showed LMP1 was highly expressed in SNK-6 cells compared with control groups. Following pretreatment with LMP1 shRNA, the proliferation of SNK-6 cells was inhibited and resulted in a G0/G1 phase arrest. A reduction in invasion and migration was also observed. LMP1 silencing promoted cell apoptosis. Further mechanistic analysis suggested that LMP1 overexpression induced the expression of eIF4E, while eIF4E-shRNA dramatically attenuated the increase in cell proliferation, invasion, migration and the inhibition of apoptosis triggered by LMP-1 upregulation. Moreover, the effect of LMP1 on eIF4E expression was mediated by the NF- κ B

pathway. Therefore, this finding may provide a potential target against NKTL.

Introduction

Nasal natural killer/T-cell lymphoma (NKTL) is a rare lymphoma that is more common in East Asia and Central America than in the West. It accounts for 7-10% of all non-Hodgkin's lymphomas diagnosed in East Asia and Latin America, but only 1% of such lymphomas among Caucasians (1-3). Among the risk factors for NKTL development, Epstein-Barr virus (EBV) latent infection has been shown to play an important role, and ENKTL is closely associated with EBV infection (4-7).

EBV is a ubiquitous herpes virus that is linked to multiple malignancies, including Burkitt's lymphoma, Hodgkin's disease, gastric, esophageal, cervical and prostate cancer and nasopharyngeal carcinoma (NPC) (8-12). Latent membrane protein 1 (LMP1) encoded by EBV functions as an essential factor in EBV-induced cell transformation and is expressed in many of the malignancies associated with EBV (13). Clinical studies showed that the expression of LMP1 was significantly correlated with the prognosis of patients with ENKTL. Studies of LMP1 in nasopharyngeal carcinoma indicated that LMP1 can enhance nasopharyngeal carcinoma cell migration and invasion. Moreover, the human Fab-based immune-conjugate specific for the LMP1 extracellular domain can inhibit nasopharyngeal carcinoma growth both *in vitro* and *in vivo* (14,15). These findings suggest that LMP1 may play an important role in the progression of ENKTL. Therefore, investigation of the effect and mechanism of action of LMP1 on ENKTL cells may reveal a potential target for ENKTL treatment.

In the present study, we analyzed the expression of LMP1 in an NKTL cell line (SNK-6) and aimed to assess the effect of LMP1 on NKTL progression. After transfection with LMP1 shRNA, cell proliferation, migration and invasion were analyzed. The underlying mechanism was also analyzed.

Materials and methods

Antibodies and reagents. Unless stated otherwise, all substances were purchased from Gibco (Grand Island, NY, USA). RPMI-1640 medium was purchased from Sigma (St. Louis, MO, USA). Rabbit anti-LMP-1, eIF4E, NF- κ B and I κ B monoclonal

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Abbreviations: NKTL, nasal natural killer T-cell lymphoma; EBV, Epstein-Barr virus; LMP1, Latent membrane protein1; eIF4E, eukaryotic translation initiation factor 4E; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PI, propidium iodide; NPC, nasopharyngeal carcinoma; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate

Key words: nasal natural killer T-cell lymphoma, SNK-6 cell, LMP1, eIF4E

antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β -actin antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). HRP-conjugated goat anti mouse and anti-rabbit antibodies were from Abcam (Cambridge, MA, USA).

Cell lines and cell culture. The SNK-6 cell line was purchased from ScienCell (San Diego, CA, USA). Cells were cultured in RPMI-1640 media supplemented with 10% heat-inactivated human plasma, 700 U/ml of recombinant interleukin-2 (IL-2; Novartis, Surrey, UK), 50 U/ml penicillin and 50 μ g/ml streptomycin. PBMCs from healthy volunteers were isolated by centrifugation using Ficoll-Hypaque (Amersham Pharmacia Biotech, Buckinghamshire, UK). Both cell lines were incubated at 37°C in an atmosphere containing 5% CO₂. Cells used for the experiments were all in the logarithmic phase.

Infection of SNK-6 cells with lentiviral LMP1/eIF4E shRNA expression vector. The shRNA-LMP1/eIF4E and its control shRNA-NC plasmids were designed and synthesized by Shanghai GeneChem Chemical Technology Co., Ltd. (Shanghai, China). The stable LMP1/eIF4E knocked down cell line was established via lentiviral vector transfection. SNK-6 cells in the logarithmic growth phase were cultured with lentiviral vector solution for 6 h, and supplemented with lentiviral vector for another 6 h. After 48 h, cells were selected with hygromycin B until positive cells were identified.

LMP1 expression vector transfection. The pcDNA3.1-LMP1 plasmid was constructed by inserting the cDNA fragment retro transcribed from the full-length cDNA of LMP1 (16). Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was used for the transfection of LMP1 plasmid (pcDNA3.1-LMP-1) and empty plasmid (pcDNA3.1) according to the manufacturer's protocol. Stable expression clones were selected using G418 (neomycin sulfate, 800 μ g/ml). The cell culture medium was replaced with fresh G418-containing medium every 2-3 days until resistant colonies were identified. The cells were collected for further analysis.

MTT assay. Cell viability was monitored via the 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) colorimetric assay. Briefly, after adding 20 μ l of MTT (5 mg/ml) to each well, and a 4-h incubation at 37°C, MTT crystals were dissolved with dimethyl sulfoxide (DMSO) without discarding the cell supernatants and the absorbance (490 nm) was measured. All experiments were repeated at least three times with 96-wells per experiment.

Cell cycle and apoptosis analysis by flow cytometry. For cell cycle analysis, SNK-6 cells were cultured in 0.01 mmol/l doxorubicin, or not. After 24 h, the cells were stained in 50 mg/ml propidium iodide, 0.05% Triton X-100, 0.1 mg/ml RNase A, and 1X PBS at 37°C for 30 min in the dark. The stained cells were suspended in 500 ml PBS for flow cytometric analysis.

Cell apoptosis was analyzed using an Annexin V-PI apoptosis detection kit (Abcam, Cambridge, UK). Briefly, transfected cells were washed with PBS and resuspended in 500 μ l of binding buffer containing Annexin V-fluorescein

isothiocyanate (FITC) and propidium iodide (PI). After incubation on ice for 10 min, cells were analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). The relative number of apoptotic cells was calculated.

Transwell migration and invasion assay. The BioCoat Matrigel Invasion Chamber was purchased from BD Biosciences (Bedford, MA, USA). The membrane has a pore size of 8 μ m and is coated with Matrigel matrix. According to the manufacturer's instructions, 1x10⁶ SNK-6 cells in 0.5-ml culture medium were implanted into the upper chamber. The bottom well contained 0.6 ml of Dulbecco's modified Eagle's medium with 10% fetal bovine serum. After being cultured for 48 h, the cells in the upper and lower chambers were stained with 0.4% trypan blue and counted with a hemocytometer. After air-drying, the membrane was stained with crystal violet. The number of migrating cells was then counted under a microscope.

An invasion assay was carried out similarly to the cell migration assay, except that 0.1 ml of Matrigel (50 mg/ml; BD Biosciences) was added to the membrane surface of the chamber 6 h before the cells were seeded.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR). Cells were harvested after transfection or 48 h later. Total RNA of the cells was extracted using TRIzol reagent (Invitrogen). The cDNA was synthesized from 5 μ g of the total RNA using M-MLV reverse transcriptase (Clontech Laboratories, Palo Alto, CA, USA). The obtained cDNA was then used as a template for qRT-PCR analysis. First strand cDNA was synthesized from 2 μ g of total RNA using the SuperScript II reverse transcriptase (Invitrogen) with 200 ng of random hexamers. The qRT-PCR cycling conditions were as follows: 95°C for 2 min for initial denaturation; 94°C for 15 sec, 58°C for 15 sec, and 72°C for 20 sec; 2 sec for plate reading for 40 cycles; and melt curve from 65 to 95°C. The primers used were as follows: LMP-1, 5'-GGT ACC TAC ATA AGC CTC TCA CAC TG-3' (forward primer) and 5'-TCT AGA GAA GGT AAG AGT GCC ATC-3' (reverse primer); eIF4E, 5'-GGG CCC ATG GCG ACT GTC GAA CCG GA-3' (forward primer) and 5'-CTC GAG TTA GTG GTG GAG CCG CTC TTA-3' (reverse primer); β -actin, 5'-CTG GGA CGA CAT GGA GAA AA-3' (forward primer) and 5'-AAG GAA GGC TGG AAG AGT GC-3' (reverse primer).

Western blot analysis. After transfection for 48 h, cells were harvested after being washed with cold PBS (Invitrogen). Total protein was extracted using the RIPA lysis buffer system (Santa Cruz Biotechnology, Dallas, TX, USA). A micro-BCA protein assay kit (Pierce, Rockford, IL, USA) was used for protein concentration analysis. Total protein (30 μ g/lane) was resolved on a 12% SDS/PAGE under denatured reducing conditions and transferred onto a nitrocellulose membrane (Amersham Pharmacia, Freiburg, Germany). After 1-h incubation in blocking solution (5% non-fat milk), the membrane was probed overnight at 4°C with primary antibodies [LMP1 (1:500), eIF4E (1:300), NF- κ B (1:400), I κ B (1:400), β -actin (1:500)]. The membranes were washed five times then incubated with HRP-conjugated secondary antibodies (1:2,000) for 1 h at room temperature. Chemiluminescent detection was

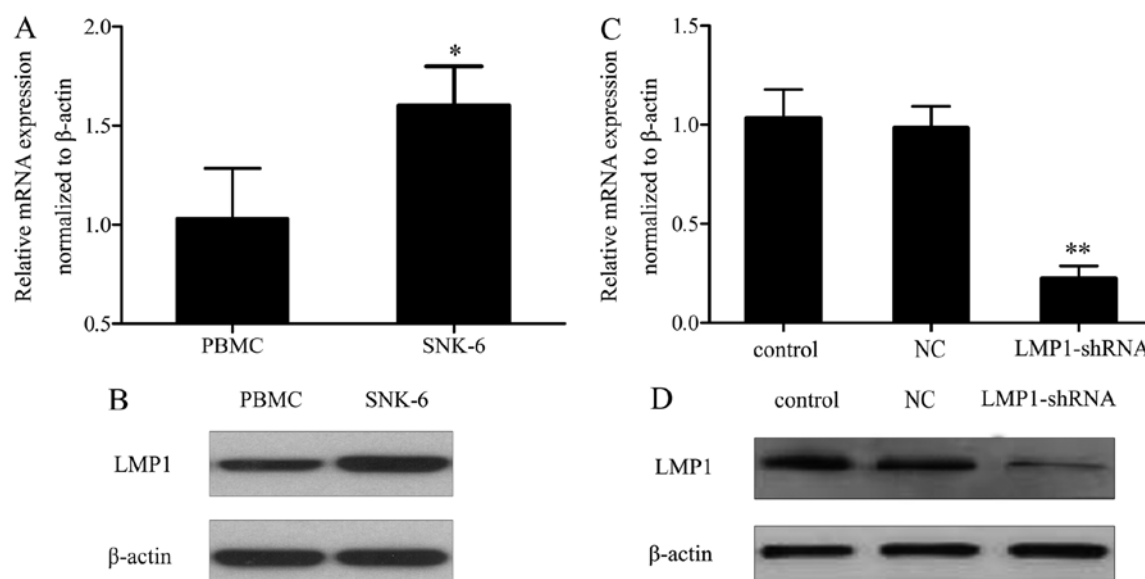


Figure 1. LMP1 is overexpressed in SNK-6 cells and effectively silenced by transfection with LV-LMP1 shRNA. (A) The mRNA level of LMP1 in SNK-6 cells was measured via qRT-PCR; (B) protein expression of LMP1 in SNK-6 cells using western blot analysis; (C) effect of LMP1 shRNA on LMP1 mRNA and (D) protein expression. β-actin served as the loading control. Results presented are mean ± SD; statistical differences from their respective controls are shown at **P<0.01 and *P<0.05, respectively.

performed using an ECL kit (Pierce Chemical, Rockford, IL, USA). The gray value of the bands was analyzed using Image J2x software.

Statistical analysis. Each experiment was repeated at least three times. The results are presented as the mean ± standard deviation (SD). Statistical analyses were performed using an independent samples Student's t-test for direct two-group comparisons and the Tukey-Kramer test after a significant one-way analysis of variance (ANOVA) F-test for multiple-group comparisons. A difference was considered statistically significant at P<0.05.

Results

LMP1 is overexpressed in SNK-6 cells. It has been demonstrated that LMP1 is associated with the development of malignancies (17,18). To investigate its function in NKTL progression, its levels were measured. RT-PCR analysis showed that the mRNA levels of LMP1 were significantly higher in SNK-6 cells than in controls (Fig. 1A). A similar result was observed for protein levels (Fig. 1B).

LMP1 shRNA inhibits SNK-6 cell proliferation and G0/G1 phase arrest. To evaluate the effect of LMP1 on NKTL development, LV-LMP1-shRNA transfection was performed. After the selection of positive cells, LMP1 mRNA and protein expression were detected by qRT-PCR and western blotting. A statistically significant decrease in LMP1 levels was observed after LMP1 shRNA transfection, compared with the NC group (Fig. 1C and D). Following transfection with LMP1 shRNA for 48 h, an obvious decrease in cell proliferation rate was observed, with a 23.0% inhibition of proliferation compared to control cells (Fig. 2A). Given that LMP1 knockdown attenuated SNK-6 cell proliferation, we next performed cell cycle

analyses on SNK-6 cells transfected with LMP1 shRNA vs. a control vector. Further flow cytometry assay showed that in comparison to the NC group, cells transfected with LMP1 shRNA induced reproducible and highly significant G0/G1 arrest. The distribution of transfected cells in the cell cycle increased by 13.4±5.7% in the G0/G1 phase (P<0.01) and decreased by 21.3±7.2% in the S phase (P<0.01) compared with controls. These results suggest that G0/G1 arrest may be the mechanism through which LMP1 regulates SNK-6 cell growth (Fig. 2B).

LMP1 shRNA suppresses SNK-6 cell migration and invasion. We further analyzed whether LMP1 shRNA pretreatment could influence the chemotaxis of SNK-6 cells. SNK-6 cell migration and invasion were assessed using a Transwell assay. As shown in Fig. 2C and D, after transfection with LMP1 shRNA, the migration and invasive potential of SNK-6 cells was markedly reduced, by 73.8±8.2 and 42.3±7.1% compared with the controls, respectively.

LMP1 shRNA promotes SNK-6 cell apoptosis. The apoptosis of differently treated SNK-6 cells was measured by FCM. The results showed that in parallel with the control groups, there was a significant increase in apoptosis, with LMP1 shRNA increasing cell apoptosis by 25.9±6.2% (Fig. 2E). This result indicates that LMP1 may inhibit cellular apoptosis in NKTL.

LMP1 promotes the expression of eIF4E in SNK-6 cells. Increased eIF4E expression is associated with enhanced invasion and metastasis in many kinds of tumors (ref.?). To clarify the underlying mechanism involved in LMP1 regulated SNK-6 cell proliferation, migration, invasion and apoptosis, the expression of eIF4E was analyzed. As expected, after transfection with pcDNA3.0 or pcDNA3.0-LMP1 for 48 h, qRT-PCR analysis showed that compared with the control

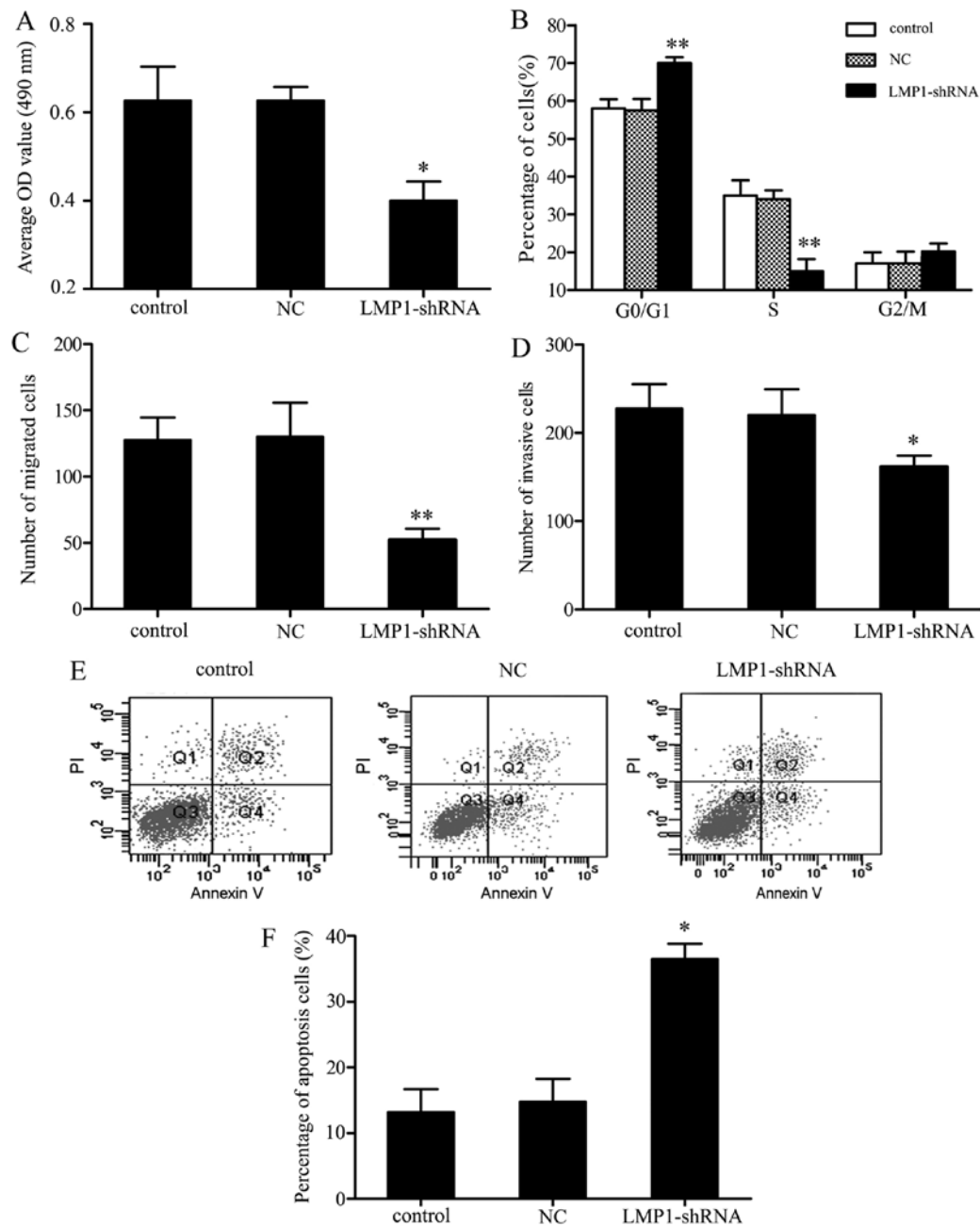


Figure 2. LMP1 regulates SNK-6 cell function. (A) MTT assays revealed that silencing of LMP1 repressed the proliferation of SNK-6 cells compared to the negative control (NC) group; (B) LMP1 knockdown induced G0/G1-phase arrest in SNK-6 cells; (C) effects of LMP1 shRNA on the migration and (D) invasion of SNK-6 cells. Average number of invasive and migrated cells from three separate experiments. (E) Apoptosis of SNK-6 cells when LMP1 was knocked down; (F) quantitative description of cell apoptosis in SNK-6 cells. Data presented are mean \pm SD. Statistical differences from their respective controls are shown at ** $P < 0.01$ and * $P < 0.05$, respectively.

group, pcDNA3.0-LMP1 transfection increased the mRNA level of eIF4E by ~ 1.5 -fold (Fig. 3A). Further analysis of LMP1 and eIF4E protein expression by western blotting showed that along with the increased protein level of LMP1, eIF4E expression was also upregulated compared with controls, suggesting that LMP1 tended to increase eIF4E expression (Fig. 3B and C).

eIF4E is responsible for LMP1-induced SNK-6 cell proliferation, migration, invasion and apoptosis. To further analyze whether LMP1 regulated SNK-6 cell function via eIF4E expression, we performed a series of functional restoration

assays. Following pretreatment with eIF4EshRNA, cells were then transfected with pcDNA3.0-LMP1, and the corresponding effect on cell proliferation, migration, invasion and apoptosis was assessed as before. The results showed that eIF4E silencing decreased the proliferation (Fig. 4A), migration (Fig. 4B), and invasion (Fig. 4C) of SNK-6 cells induced by LMP1 upregulation. The inhibitory effect on apoptosis triggered by LMP1 overexpression was also ameliorated in the eIF4E-silenced groups (Fig. 4D and E). These results suggested that LMP1 may trigger the proliferation, migration, invasion and apoptosis of SNK-6 cells by increasing eIF4E expression.

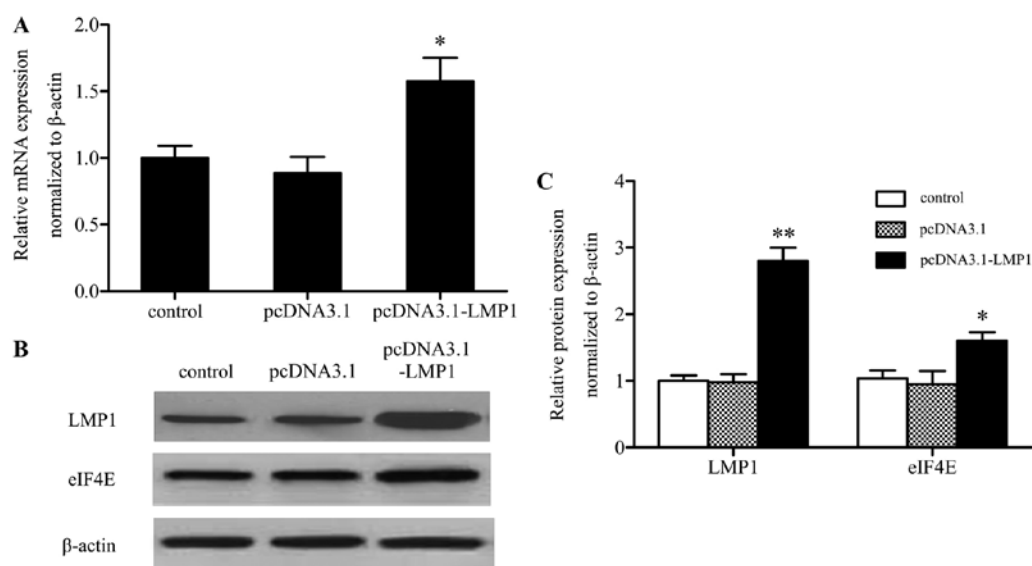


Figure 3. LMP1 stimulates eIF4E expression in SNK-6 cells. (A) After transfection for 48 h, the mRNA levels of eIF4E were confirmed by qRT-PCR. The corresponding protein expression was measured by (B) western blotting and (C) quantified. β -actin served as the loading control. Results presented are mean \pm SD; statistical differences from their respective controls are shown at ** $P < 0.01$ and * $P < 0.05$, respectively.

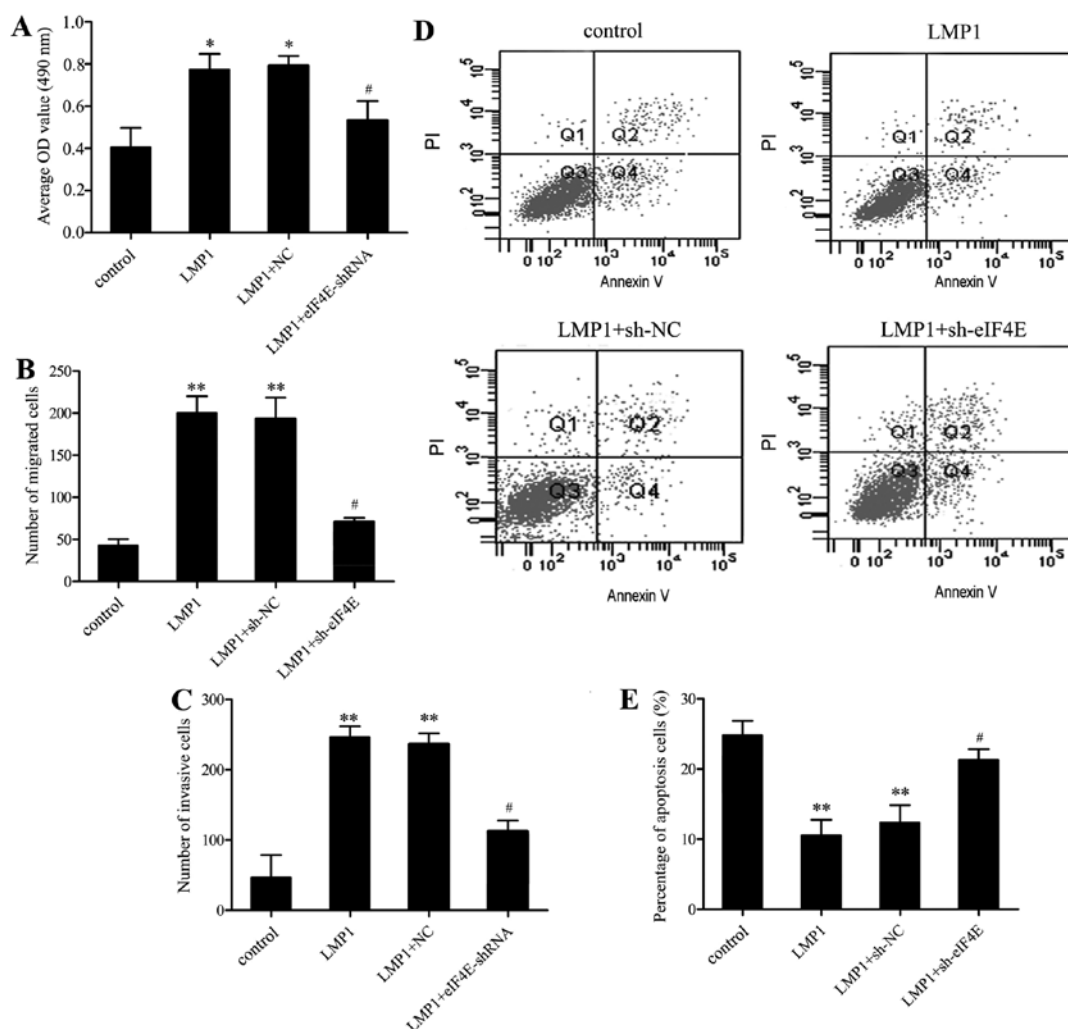


Figure 4. LMP1 regulates the biological behavior of SNK-6 cells partly dependent on eIF4E expression. After pretreatment with pcDNA3.0-LMP1 and eIF4E shRNA, the (A) proliferation, (B) migration, (C) invasion and (D and E) apoptosis of SNK-6 cells were assessed. LMP1: group transfected with the LMP1 expression vector pcDNA3.0-LMP1, shNC cells stably infected with the negative control lentiviral shRNA vector, or eIF4E-shRNA cells stably infected with the lentiviral vector of shRNA targeting eIF4E, respectively. Data presented are mean \pm SD. Statistical differences from controls are shown at ** $P < 0.01$ and * $P < 0.05$; statistical differences from LMP1 group are shown at # $P < 0.01$.

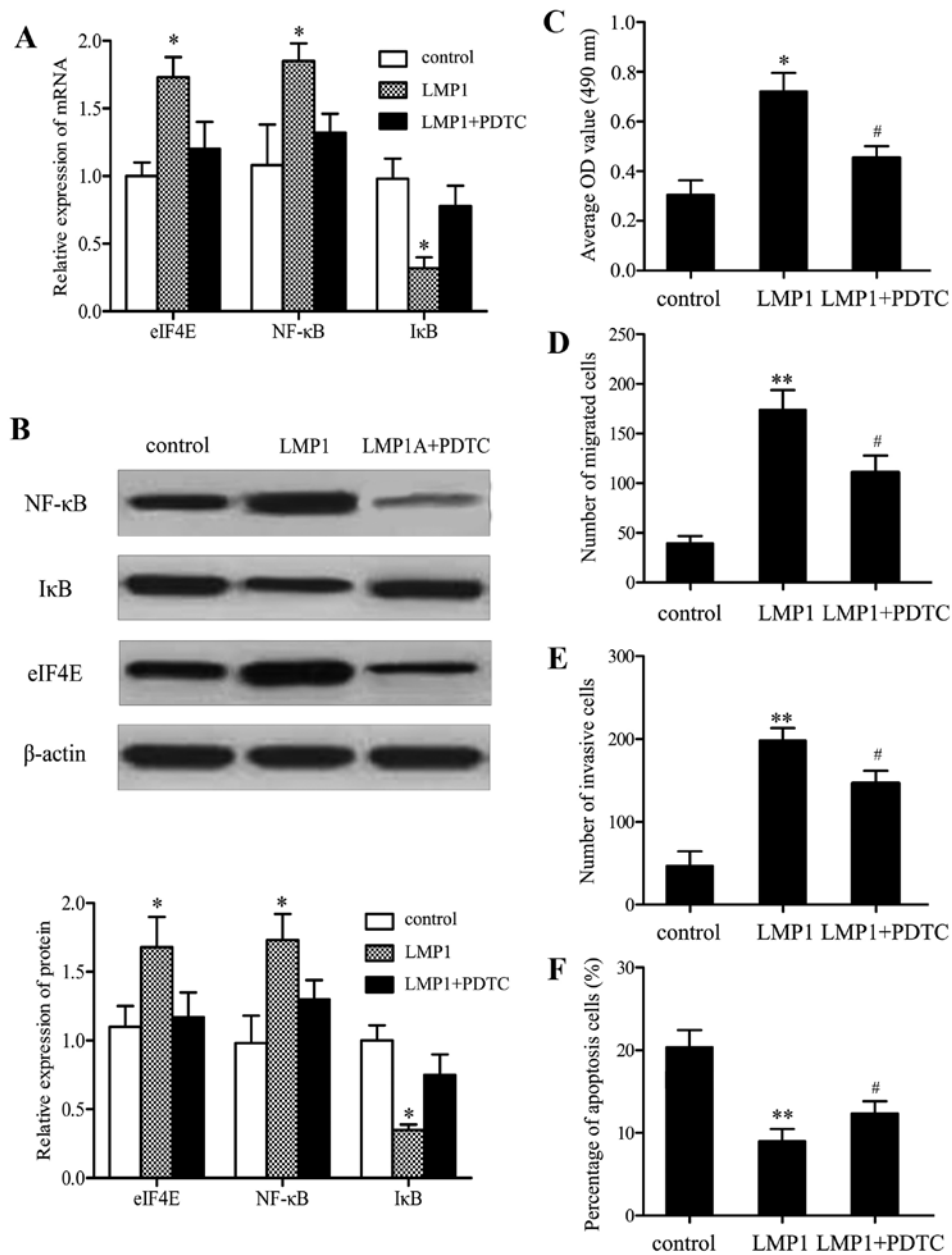


Figure 5. LMP1 induces eIF4E expression through the NF- κ B pathway in SNK-6 cells. (A) After pretreatment of SNK-6 cells with the NF- κ B signaling pathway inhibitor PDTC for 48 h, the mRNA level of eIF4E was analyzed by qRT-PCR. (B) Corresponding protein expression was assessed by western blotting. The (C) proliferation, (D) migration, (E) invasion and (F) apoptosis of SNK-6 cells were also assessed. β -actin served as the loading control. Results presented are mean \pm SD; statistical differences from their respective controls are shown at ** $P < 0.01$ and * $P < 0.05$, respectively; statistical differences from LMP1 group are shown at # $P < 0.05$.

Effect of LMP1 on eIF4E expression is mediated by the NF- κ B pathway. To further explore the underlying mechanism involved in LMP1-induced eIF4E expression, the NF- κ B pathway was investigated. As expected, LMP1 overexpression clearly induced activation of the NF- κ B pathway (Fig. 5A). However, following pretreatment with pyrrolidinedithiocarbamate (PDTC; Sigma), an inhibitor of the pathway, the expression of eIF4E induced by LMP1 was dramatically downregulated. Similar effects on eIF4E protein levels were observed, indicating that LMP1 induces eIF4E expression by activating the NF- κ B pathway (Fig. 5B). SNK-6 cell proliferation, migration, invasion, and apoptosis were also assessed in order to certify whether the effect of LMP1 on SNK-6 cell function was mediated by the NF- κ B pathway. MTT analysis showed that after

pretreatment with PDTC, the proliferation induced by LMP1 was effectively attenuated (Fig. 5C). Moreover, the results of the transwell assay indicated that the changes in cell migration (Fig. 5D) and invasion (Fig. 5E) were consistent with proliferation. PDTC pretreatment enhanced cell apoptosis significantly compared with the LMP1 group (Fig. 5F). The results demonstrated that eIF4E is responsible for LMP1-induced SNK-6 cell proliferation, migration, invasion and apoptosis via the NF- κ B signaling pathway.

Discussion

NKTL is an uncommon disease, usually showing a highly aggressive clinical course (19). Improved understanding of this

deadly cancer at the basic molecular level is greatly needed. The present study provides insight into a potential regulatory mechanism involved in NKTL.

NKTL is universally associated with EBV infection, while LMP1 is known as a major viral oncogene in the EBV carcinogenic process (20). However, the effect of LMP1 on NKTL is not clear. Our data confirmed that LMP1 is highly expressed in the NKTL cell line SNK-6 compared to PBMCs. The findings indicate that LMP1 might play an important role in NKTL progression. Further functional analysis revealed that transfecting LMP1 shRNA1 into SNK-6 cells induces slower proliferation, migration, and invasion with cell cycle arrest at the G0/G1 phase, and silencing of LMP1 also promoted apoptosis in SNK-6 cells. The opposite effect was seen, when cells were transfected with pcDNA3.0-LMP1, as expected. Those findings suggest that LMP1 may act as an oncogene in NKTL progression.

Eukaryotic translation initiation factor 4E (eIF4E) is a recently discovered oncogene (21). As a key factor in the translation initiation complex, eIF4E plays a rate limiting role in the initiation of translation of many mRNAs of oncogenes and growth factor genes (22,23). Several clinical studies have reported that the expression of eIF4E is increased in lung, breast and esophageal cancer, among others, and is closely associated with invasion and metastasis in these tumors (22,24,25). Silencing of eIF4E expression by siRNA or antisense polynucleotide reduces proliferation and changes the cell cycle of laryngocarcinoma, esophageal carcinoma, and breast carcinoma (26-28). To explore whether eIF4E is associated with LMP1-induced cell function changes or plays a role in NKTL progression, we transfected SNK-6 cells with LMP1, and found that LMP1 significantly activated the transcriptional activity of eIF4E in SNK-6 cells, indicating that LMP1 might affect NKTL progression by activating the transcription of eIF4E. We then transfected SNK-6 cells with LV-eIF4E-shRNA and found that the expression of eIF4E decreased. Further mechanistic analysis showed that blocking eIF4E expression significantly reduced SNK-6 cell proliferation, migration, and invasion induced by LMP1 overexpression, and increased cell apoptosis following eIF4E silencing. The results confirmed that LMP1 may regulate the biological function of SNK-6 cells by enhancing eIF4E expression. However, the mechanism by which LMP1 enhances the expression of eIF4E in NKTL remains unclear.

A previous study proved that LMP1 increased the development of lymphoma in LMP1 transgenic mice through the NF- κ B pathway (29). In addition, eIF4E is a direct transcriptional target of NF- κ B and is aberrantly regulated in acute myeloid leukemia (30). Thus, the role of NF- κ B in the induction of eIF4E expression by LMP1 was evaluated in the present study. As expected, LMP1 overexpression induced activation of the NF- κ B pathway. Furthermore, eIF4E expression induced by LMP1 overexpression was inhibited by PDTC, an inhibitor of NF- κ B, indicating that LMP1 may increase eIF4E expression via the NF- κ B signaling pathway. Further functional analysis demonstrated that pretreatment with PDTC, ameliorated the effect on cell proliferation, invasion and migration triggered by LMP1, and was accompanied by increased cell apoptosis. Together, these results suggest that

LMP1 may act as an oncogene in NKTL by regulating eIF4E via activating the NF- κ B pathway.

In summary, we have shown that LMP1 is overexpressed in NKTL and that silencing it inhibits cell proliferation, cell-cycle progression, migration, invasion and promotes apoptosis. Furthermore, we suggest that LMP1 regulated the development of NKTL by regulating eIF4E expression via the NF- κ B signaling pathway. This might be a major mechanism for NKTL and other EBV-associated tumors. Therefore, the present study provides insight into the underlying mechanism by which LMP1 regulates the progression of NKTL and provides a new target for treatment of NKTL.

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