Berberine inhibits the proliferation of human nasopharyngeal carcinoma cells via an Epstein-Barr virus nuclear antigen 1-dependent mechanism

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Abstract. Nasopharyngeal carcinoma (NPC) is a malignancy derived from the epithelial cells of the nasopharynx cavity, and is closely associated with Epstein-Barr virus (EBV) infection. In addition to NPC, EBV causes various human malignancies, such as gastric cancer, hematological tumors and lymphoepithelioma-like carcinomas. Epstein-Barr nuclear antigen 1 (EBNA1) encoded by EBV is indispensable for replication, partition, transcription and maintenance of viral genomes. Berberine, a naturally occurring isoquinoline alkaloid, shows anti-inflammatory, anticholinergic, antioxidative, and anticancer activities. In the present study, the antitumor effect of berberine was studied. Cell Counting Kit-8 (CCK-8) assays were performed to demonstrate whether the proliferation of EBV-positive NPC cells was inhibited by berberine. Flow cytometric results revealed that berberine induced cell cycle arrest and apoptosis. Quantitative-PCR and western blotting results indicated that berberine decreased the expression of EBNA1 at both the mRNA and protein levels in the EBV-positive NPC cells. The function of EBNA1 promoter Qp which is to drive EBNA1 transcription in type II latent infection was strongly suppressed by berberine. Overexpression of EBNA1 attenuated this inhibitory effect. Berberine also suppressed the activity of signal transducer and activator of transcription 3 which is a new therapeutic target in a series of malignancies, including NPC. Viral titer experiments demonstrated that berberine decreased the production of virions in HONE1 and HK1-EBV cells. In a mouse xenograft model of NPC induced by HONE1 cells, berberine significantly inhibited tumor formation. Altogether, these results indicate that berberine decreases the expression of EBNA1 and exhibits an antitumor effect against NPC both *in vitro* and *in vivo*.

Introduction

The Epstein-Barr virus (EBV) is a common γ-herpesvirus with a high prevalence in adults worldwide (1). EBV infection is considered a high risk factor and is responsible for more than 1% of all human cancers (2). EBV infection is associated with various human proliferative diseases involving mostly epithelial or lymphoid cells, including nasopharyngeal carcinoma (NPC), gastric and lymphoepithelioma-like carcinomas (3-5). NPC occurs in the epithelium of the throat and nose and can be classified as two broad subtypes, i.e. keratinized (differentiated epithelial) and non-keratinized (undifferentiated epithelial) cells (6). NPC is prevalent in areas such as southern China, Southeast Asia, Alaska and native populations of Canada (7). EBV exhibits a type II latency mechanism in NPC patients (8-10). Type II latency infection is characterized by the expression of viral Epstein-Barr nuclear antigen 1 (EBNA1), latent membrane proteins (LMP1, LMP2A and LMP2B), and several EBV non-coding RNAs (11,12). The expression of special viral antigens in NPC associated with EBV make this disease an attractive target for the therapy of NPC (9).

EBNA1 is the only viral protein expressed in all three forms of latent EBV infections (13). As an important regulator of EBV latency, EBNA1 has many functions influencing the EBV genome and host cells. For example, EBNA1 plays an essential role in the maintenance and replication of the EBV genome via sequence-specific binding to the viral origin of replication (oriP) (14). EBNA1 has been shown to modulate several cellular signaling pathways to regulate cell growth and transformation, such as nuclear factor-κB (NF-κB), transforming growth factor-β (TGF-β), and signal transducer and activator of transcription (STAT) (15-17). EBNA1 also decreases the accumulation of p53 in EBV-infected epithelial cells, leading to disruption of the antitumor functions (18,19). In NPC cell type II infection, EBNA1 transcription is mediated

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by the Q promoter (Qp) (20,21). Previous studies have shown that Janus kinase (JAK)-STAT activates Qp-driven EBNA1 expression (22). Moreover, signal transducer and activator of transcription 3 (STAT3) is likely to be the biologically relevant STAT for EBNA1Qp and LMP1 L1-TR promoter regulation (23). Aberrant activation of STAT3 may be a necessary event for EBV-associated malignancies (24). In addition, increasing evidence has demonstrated that EBNA1 may be essential for the proliferation of tumor cells (25,26). Collectively, EBNA1 has become an attractive target for therapeutic strategies.

Berberine is a natural compound belonging to the alkaloids, which is present in the rhizome, roots and stem bark of a number of medicinal plants such as *Cortidis rhizome* (Huanglian), *Coptis chinensis* (Chinese goldthread), *Scutellaria caicalensis* (Baikal Skullcap) and *Berberine vulgaris* (barberry) (27,28). Berberine is found to have multiple pharmacological functions such as anti-diarrheal, anti-hypertensive, anti-microbial and anti-inflammatory effects (29-32). A previous study showed the potential cancer chemopreventive effect of berberine (33). Moreover, berberine has been reported to inhibit the growth of various types of cancer, such as NPC, non-small lung cancer, primary effusion lymphoma, and breast and liver cancer (34). Berberine was found to inhibit the proliferation of human breast cancer cells (MCF-7) through the down-regulation of the expression of various tumor-related proteins including HER2, Bcl-2, COX-2 and EGFR, and the upregulation of p21 and IFN-α expression (35-38). A previous study showed that berberine induced mitochondrial apoptosis in EBV-transformed B cells through the upregulation of XAF1 and GADD45α expression by MAPK and functional p53 (39). Various findings demonstrated that berberine induces the apoptosis of NPC cells through the downregulation of the activity of STAT3 (40).

Our laboratory previously demonstrated that heat shock protein 90 (Hsp90) inhibitors blocked outgrowth of EBV-infected malignant cells through an EBNA1-dependent mechanism *in vitro* and *in vivo* (41). Recently, we found that triptolide inhibited the proliferation of EBV-positive B lymphocytes via the inhibition of LMP1 (42). Moreover, our laboratory demonstrated that triptolide, a diterpenoid triepoxide purified from the roots of Chinese herb *Tripterygium wilfordii*, showed antitumor activity against Kaposi's sarcoma-associated herpesvirus (KSHV)-related primary effusion lymphoma through a KSHV latency-associated nuclear antigen 1-dependent mechanism (43). KSHV and EBV, members of the γ-herpesvirus subfamily, are very similar. Furthermore, in addition to inhibiting the human telomerase reverse transcriptase (hTERT) transcription and protein expression, triptolide decreased the stability of hTERT in KSHV-positive BCBL-1 and BC-3 cells (44).

In the present study, we demonstrated that berberine effectively inhibited cell proliferation and induced cycle arrest and apoptosis of EBV-positive NPC cells. Berberine decreased EBNA1 transcription by inhibiting the activation of EBNA1 promoter Qp. Berberine inhibited the expression of p-STAT3 and overexpression of EBNA1 attenuated the inhibitory effect of berberine. In addition, the production of virions were decreased by berberine. Moreover, our *in vivo* results revealed that tumor growth of EBV-positive NPC in non-obese

diabetic/severe-combined immunodeficient (NOD/SCID) mice was effectively inhibited by a non-toxic dose (27,45) of berberine. These findings may provide insights into the underlying mechanism involved in the inhibition of NPC cell proliferation by berberine.

Materials and methods

Cell lines and reagents. EBV-positive NPC cell lines HONE1 and HK1-EBV were kindly provided by Professor Sai Wah Tsao (The University of Hong Kong, Hong Kong, China). HeLa cells were obtained from Professor Hui Li (Wuhan University, Wuhan, China). The EBV-negative HK2 cells were kindly provided by Professor Ling Zheng (Wuhan University).

HONE1 and HK1-EBV cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD, USA) and G418 (400 ng/ml). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. HK2 cells were cultured in DME/F-12 containing 10% FBS and hEGF (Sigma-Aldrich, Shanghai, China). All cell lines were cultured at 37˚C with a humidified atmosphere of 5% CO_2. Berberine chloride, cyclo-heximide (CHX) (both from Sigma-Aldrich, Shanghai, China), acyclovir (ACV; Selleck Chemicals, Shanghai, China), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO). Sodium butyrate (SB; Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS).

Plasmids. Plasmid pSG5-EBNA1 was constructed by ligating the *Eco*RI-*Xba*I fragment, which contains the EBNA1 sequence, into the pSG5 vector (YouBio, Shanghai, China). Plasmid pGL3.0-basic was purchased from Promega (Madison, WI, USA). PRL-TK, a plasmid expressing *Renilla* luciferase, was kindly provided by Professor Deyin Guo (Wuhan University). The sequence of Qp was amplified from the HONE1-strain EBV genome sequence. The primers used in the present study were as follows: Qp forward, 5'-TCAGATCT TATAACGCAGGTCCTG-3' and reverse, 5'-CGCAAGCTTT GTAAGGATAGCATG-3' (YouBio). The amplified sequence was digested with *Bgl*II and *Hind*III (NEB, Ipswich, MA, USA). All plasmids were purified through columns (Axygen Scientific Inc., Union City, CA, USA) as described by the manufacturer and confirmed by DNA sequencing.

Cell viability assay. The viability of the cells was determined by the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). Briefly, HONE1 and HK1-EBV cells were placed into 96-well plates with DMEM at a density of $1x10^4$ cells/100 μl. All cells were treated with controls (DMSO; 0.006%, vol/vol) and increasing concentrations of berberine for 24 and 48 h. Tetrazolium substrates were added into each well (10 μl/well) after different treatments. The plates were incubated at 37˚C for 1 h. The optical density (OD) was assessed at 450 nm with an ELx800 microimmuno-analyser (BioTek Instruments, Inc., Winooski, VT, USA).

Flow cytometric analysis. Apoptosis was quantified using an Annexin V-FITC/propidium iodide (PI) apoptosis detection

kit (MultiSciences, Shanghai, China). Briefly, HONE1 and HK1-EBV cells were placed in 6-well plates and treated with the vehicle control (DMSO; 0.006%, vol/vol) or berberine (50 or 100 μM) for 24 h. Cells were harvested, washed and re-suspended in 500 μl of binding buffer. Then, 5 μl of Annexin V-FITC and 10 μl of PI were added before being analyzed by a Beckman Coulter system (EPICS Altra II; Beckman Coulter, Fullerton, CA,USA).

Cell transfection. For transfection, all cells (HONE1, HK1-EBV and HeLa) were transiently transfected using X-tremeGENE HP DNA transfection reagent (Roche, Basel, Switzerland). At 4 h post-transfection, cells were treated with the control (DMSO; 0.006%, vol/vol) or berberine for 44 h before harvesting. CHX (50 μg/ml) was used in experiments as described in the following sections.

Dual luciferase reporter assay. In brief, HeLa cells were cultured in 48-well plates ($1x10^5$ cells/well) before transfection. After 24 h, pGL3.0-Qp (100 ng/well) and pRL-TK (5 ng/well) were co-transfected into the HeLa cells. Then, the cells were treated with the vehicle control (DMSO; 0.006%, vol/vol) or berberine (25 and 50 μM, respectively) for 24 h, at 4 h post-transfection. Subsequently, the medium was removed and cells were rinsed twice. Total proteins were harvested in 1X lysis buffer (100 μl/well). Luciferase activity was assessed by the GLO-MAX 20/20 system (both from Promega) following the manufacturer's instructions. *Renilla* luciferase was used to normalize the firefly luciferase activity.

Quantitative-PCR. The total RNA was extracted using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from the RNA using a reverse transcription kit (Takara, Tokyo, Japan) with random primers. The expression of LMP1 and EBNA1 mRNAs was quantified by CFX96 real-time PCR detection system using a SYBR *Premix Ex Taq*™ kit (Takara). The relative amounts of LMP1 and EBNA1 mRNAs were normalized to the housekeeping gene GAPDH. The primers were as follows: LMP1 forward, 5'-CTATTCCTTTGCTCTC ATGC-3' and reverse, 5'-TGAGCAGGAGGGTGATCATC-3'; EBNA1 forward, 5'-GGTCGTGGACGTGGAGAAAA-3' and reverse, 5'-GGTGGAGACCCGGATGATG-3'; GAPDH forward, 5'-ACATCGCTCAGACACCATG-3' and reverse, 5'-TGTAGTTGAGGTCAATGAAGGG-3' (YouBio). The quantitative-PCR conditions were as follows: a 30-sec denaturation at 95°C, followed by 40 cycles of 10 sec at 95°C, 10 sec at 60°C, and 20 sec at 72°C. The specificity of the reaction was controlled by melting curve analysis (65-95°C, 0.5°C/sec).

Western blotting. Cells treated under different conditions were harvested in RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with 0.5 mM phenylmethylsufonyl fluoride (PMSF) and 0.5% cocktail protease inhibitor (Roche) using a micro-scraper. After being sonicated for 15 sec, whole cell extracts were obtained by centrifugation at 12,000 x g for 15 min. The supernatants were transferred to new tubes, and the protein concentration was determined by the bicinchoninic acid method using bovine serum albumin as a standard. Equal amounts of proteins were

mixed with 5X loading buffer [250 nM Tris-HCl (pH 6.8), 0.5% BPB, 10% SDS, 50% glycerol, 5% β-mercaptoethanol]. The proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were run at 110 V, and then, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline and Tween-20 (TBST) for 1 h at room temperature, followed by incubation with the primary antibody overnight at 4°C. After 3x15 min washes with TBST, the membrane was incubated with the appropriate secondary antibodies for 1 h.

The primary antibodies used were as follows: GAPDH (cat. no. 10494-1-AP, 1:10,000; Proteintech, Wuhan, China), EBNA1 (cat. no. sc81581, 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), LMP1 (1:4,000; Abcam, Cambridge, UK), BZLF1 (1:500; Dako, Glostrup, Denmark), caspase-3 (cat. no. 9668, 1:1,000), cleaved caspase-3 (cat. no. 9664, 1:1,000), STAT3 (cat. no. 9139, 1:1,000), phospho-STAT3 (cat. no. 4113, 1:1,000, Tyr705), Mcl-1 (cat. no. 14765, 1:1,000) (all from Cell Signaling Technology Inc., Danvers, MA, USA). Secondary antibodies were horseradish peroxidase-conjugated secondary anti-mouse IgG (cat. no. 7076, 1:10,000), anti-rabbit IgG (cat. no. 7074, 1:10,000) (both from Cell Signaling Technology Inc.). Immunoreactivity was detected using the ECL system (Bio-Rad Laboratories). Band gray values were assessed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

EBV copy number analysis. HONE1 and HK1-EBV cells were induced to lytic replication phase by TPA (40 ng/ml) and SB (3 mM). After 3 h, cells were cultured in the absence or presence of berberine for 48 h. EBV virion-associated DNA was collected and amplified. The supernatants of HONE1 and HK1-EBV cells were harvested and filtered with a 0.45 μl filter. Each supernatant was incubated with 2 μl DNase I and 10X DNase I buffer (10 mM Tris-HCl, 0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$) at 37°C. After 60 min, EDTA (2 mM, 20 μl, pH 8.0) was added to inhibit the activity of DNase I. All the samples were treated with proteinase K (0.1 mg/ml) [sample:proteinase K = 1:1 (vol/vol)] for 1 h at 50°C. The reactions were stopped at 75°C after 20 min by inhibiting the activity of proteinase K (46). Subsequently, each sample and strand was examined for the sequence of EBNA1 by quantitative-PCR assay using a SYBR-Green PCR kit (cat no. RR420A; Takara Bio). Each quantitative-PCR reaction mixture included 2 μl viral DNA from the prepared sample, 2 μl specific primers (0.2 μM), 12.5 μl 2X SYBR Premix Ex Taq, 0.4 μl ROX reference dye or Dyell and PCR-grade water for a final volume of 25 μl. The primers were as follows: EBNA1 forward, 5'-GGTCGTGGACGTGGAGAAAA-3' and reverse, 5'-GGTGGAGACCCGGATGATG-3' (YouBio). The quantitative-PCR conditions were: a 5-sec denaturation at 95°C, a 20-sec annealing at 60°C, a 2-sec extension of primers at 72°C for 45 cycles. Melting curve analysis was performed from 65-95°C (with 0.1°C/sec).

In vivo tumor studies. All experimental procedures and protocols were approved by the Medical Ethics Committee of Wuhan University. The NOD/SCID mice were purchased

and maintained in the Animal Experiment Center of Wuhan University, Animal Biosafety Level-III Laboratory.

Eight-week-old female NOD/SCID mice were subcutaneously inoculated into flanks with $1x10^7$ HONE1 cells suspended in 100 μl of PBS. Seven days later, the mice (five mice/group) were treated with intraperitoneal injection of DMSO (0.006%) or berberine (10 mg/kg, three times a week). Mice were sacrificed after three weeks by cervical dislocation. Tumor size was assessed, and then the tumors were fixed in 10% neutral buffered formalin. Immunohistochemical analysis and hematoxylin and eosin (H&E) staining were performed according to the method described in a previous study (47).

Immunohistochemistry. Tumor samples were formalin-fixed and paraffin-embedded and cut into sections of 4-μm thickness. The sections were dewaxed in xylene, rehydrated in ethanol, washed in PBS, and then stained with H&E. After staining, sections were dehydrated through a series of increasing concentrations of ethanol and xylene, and rehydrated in distilled water. After antigen retrieval in a sodium citrate buffer in a microwave oven, the endogenous peroxidase activity was blocked using 0.6% H_2O_2 for 30 min. Sections were incubated overnight at 4°C with a primary antibody EBNA1 (cat. no. ab8329, 1:1,000; Abcam). After washing in PBS twice, the sample sections underwent detection using the anti-mouse-specific HRP/AEC Detection IHC kit (ab127055; Abcam) according to the manufacturer's instructions.

Statistical analysis. All results were normalized to the control and are presented as the mean ± standard deviation (mean ± SD) of at least three independent experiments. The statistical significance of the difference was evaluated by Student's t-test using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Significance was assigned at $p<0.05$.

Results

Berberine inhibits the viability of EBV-positive NPC cells. To determine whether berberine (Fig. 1A) affects the cell viability of EBV-positive NPC cells, HONE1 and HK1-EBV cells were treated with a vehicle control (0.006% DMSO) or a series of increasing concentrations of berberine (25, 50, 100 and 200 μM) for 24 and 48 h, respectively. Cell viability was determined by CCK-8 assays. As shown in Fig. 1B, berberine inhibited the cell viability of HONE1 cells in a dose- and time-dependent manner. The viability of HONE1 cells was decreased from ~10 to 90% after berberine treatment for 24 and 48 h. The 50% inhibitory concentration (IC_{50}) for HONE1 cells was 101.3 and 56.7 μM with berberine treatment at 24 and 48 h, respectively. Similar results were also found in the HK1-EBV cells treated with a series of increasing concentrations of berberine. The viability of HK1-EBV cells was decreased from 15 to 78% after 24 and 48 h of berberine treatment. The IC_{50} values were calculated and were 124.5 and 43.1 μM at 24 and 48 h, respectively, in the HK1-EBV cells treated with berberine (Fig. 1C). To further evaluate the effects of berberine in the EBV-negative cell line, HK2 cells were treated with the vehicle control (0.006% DMSO) or berberine for 24 and 48 h. As shown in Fig. 1D, treatment with a series of increasing concentrations of berberine showed a slight inhibition in the proliferation of HK2 cells at 24 and 48 h. These results suggest that berberine inhibits the cell viability of EBV-positive NPC cells efficiently, but not significantly in HK2 cells.

Berberine induces cell cycle arrest and apoptosis in EBV-positive NPC cells. To examine whether berberine induces cell cycle arrest and apoptosis, HONE1 and HK1-EBV cells were treated with the vehicle control (0.006% DMSO) or berberine (50 and 100 μM) for 24 h. Cells were collected and stained with Annexin V-FITC/PI, followed by analyses with flow cytometry. The cell cycle analysis revealed that berberine-treated HONE1 cells underwent a G2 arrest; the percentage of G2 phase cells was 1.41% in the control group, and 16.2% in the berberine-treated group (Fig. 2A). Treatment of HK1-EBV cells with berberine resulted in G1 phase arrest; the percentage of G1 phase cells was 50.9% in the control (0.006% DMSO) and 56.9% in the berberine-treated group (50 μM) (Fig. 2A). In addition, compared to the control group (0.006% DMSO), berberine (50 or 100 μM) induced cell apoptosis in the HONE1 and HK1-EBV cells (Fig. 2B). The results suggest that berberine induced cell cycle arrest and apoptosis in the EBV-positive HONE1 and HK1-EBV cells.

Berberine decreases EBNA1 expression in EBV-positive NPC cells. To determine whether berberine alters the expression of EBNA1 in EBV-positive NPC cell lines, HONE1 and HK1-EBV cells were treated with the vehicle control (0.006% DMSO) or berberine (25 or 50 μM) for 48 h. Whole-cell extracts were harvested and subjected to western blotting. As shown in Fig. 3A, the expression levels of EBNA1 in the HONE1 cells were decreased to 36.8 and 6.7%, with berberine treatment at 25 and 50 μM, respectively, when compared to the control group. HK1-EBV cells were also treated with the same concentrations of berberine. When compared to the control group, the expression levels of EBNA1 were not significantly affected in the 25 μM berberine-treated group, but downregulated to 32.4% in the 50 μM berberine-treated group. However, the expression level of another EBV protein, LMP1, was not decreased by berberine in both the HONE1 and HK1-EBV cells. Furthermore, compared to the control group, the expression levels of cleaved-caspase-3 in the HONE1 and HK1-EBV cells were increased while the levels of caspase-3 exhibited no obvious changes with berberine treatment. The different response of the HONE1 and HK1-EBV cells to berberine is possibly due to the different sensitivity of the cell lines to this drug.

In addition, quantitative PCR analysis was used to detect whether berberine affects LMP1 at the mRNA level. HONE1 and HK1-EBV cells were treated with the vehicle control (0.006% DMSO) or berberine (25 or 50 μM) for 24 h, respectively. The total RNAs were collected and subjected to quantitative-PCR. The relative expression of LMP1 was detected by comparison to the GAPDH gene. As shown in Fig. 3B, the mRNA levels of LMP1 in the HONE1 and HK1-EBV cells were not decreased by berberine.

Berberine treatment in a previous study was not observed to affect the viability of EBV-negative HeLa cells (48). Therefore, HeLa cells were used to determine whether berberine specifically decreases the expression of EBNA1 in the present study. In order to determine whether berberine specifically decreases
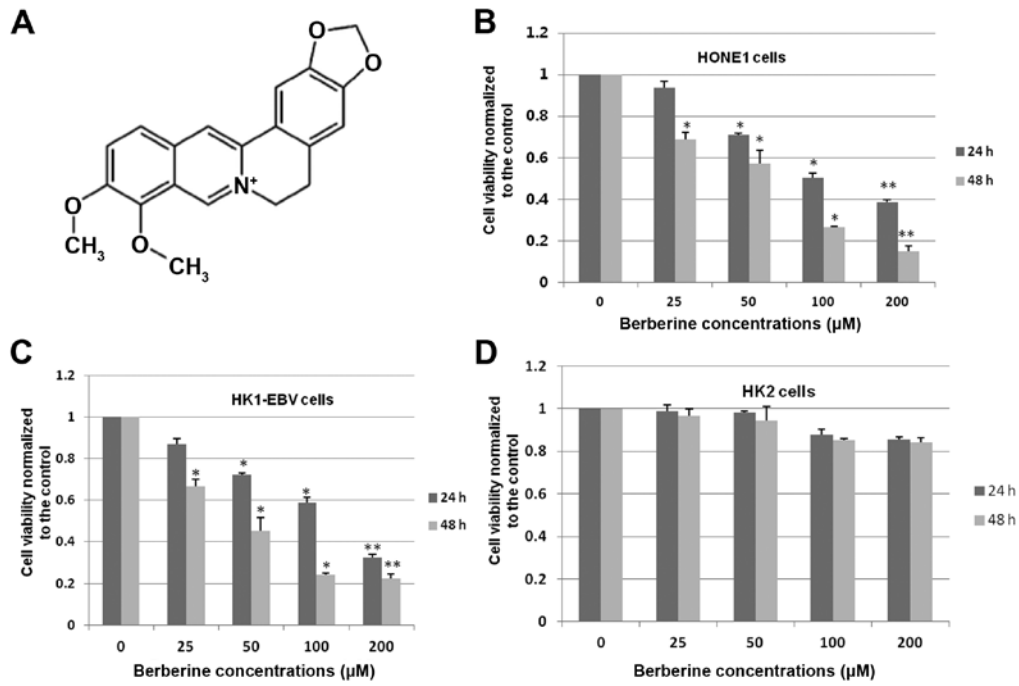
Figure 1. Berberine inhibits the proliferation of EBV-positive NPC cells. (A) Molecular structure of berberine. (B) HONE1, (C) HK1-EBV and (D) HK2 cells were treated with the vehicle control (0.006% DMSO only) or a series of concentrations of 25, 50, 100 and 200 μM of berberine for 24 or 48 h, respectively. The cell proliferation assay was carried out using a CCK-8 assay kit as described in Materials and methods. The mean values of at least three independent experiments are shown; *p<0.05; **p<0.01, compared with the vehicle control (0.006% DMSO). EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma; DMSO, dimethyl sulfoxide.
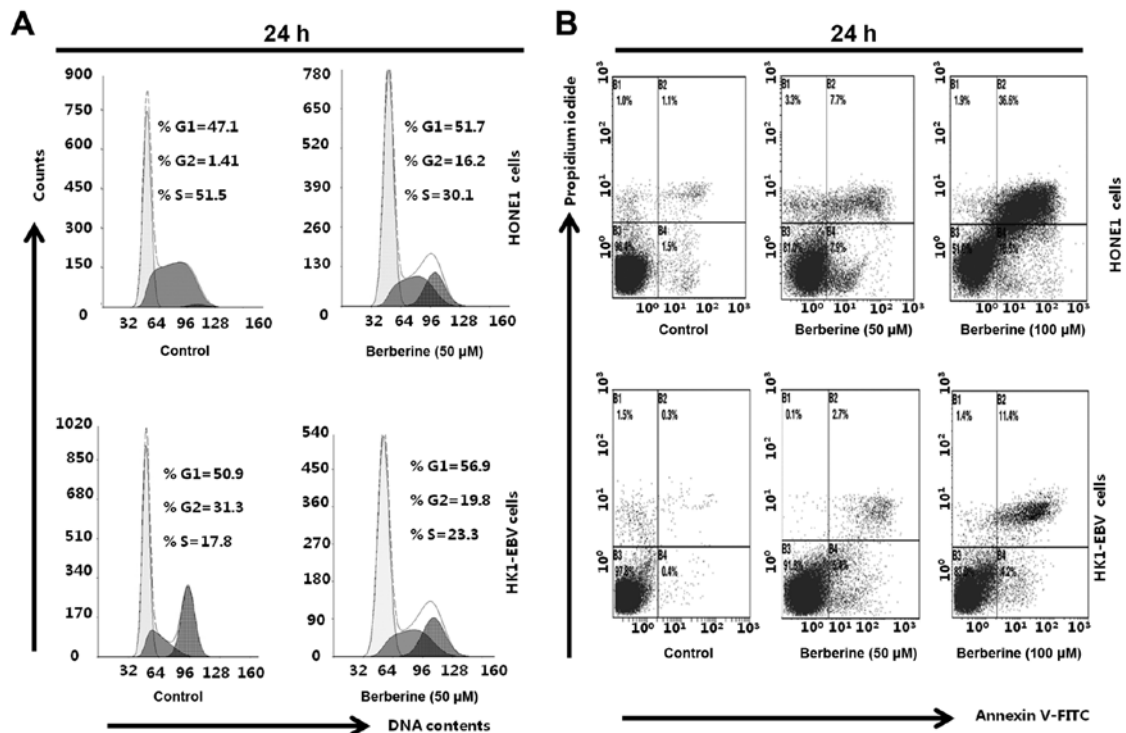

Figure 2. Berberine induces cell cycle arrest and apoptosis in the EBV-positive NPC cells. (A) The effect of berberine on the cell cycle. HONE1 or HK1-EBV cells were treated with the vehicle control (0.006% DMSO) or indicated concentrations of berberine for 24 h, respectively. The distribution of the cell cycle was determined by flow cytometry. (B) The effect of berberine on apoptosis. HONE1 or HK1-EBV cells were treated with the vehicle control (0.006% DMSO) or indicated concentrations of berberine for 24 h. Apoptotic cells were determined by flow cytometry. EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma; DMSO, dimethyl sulfoxide.

the expression of EBNA1, p-SG5 and pSG5-EBNA1 were transiently transfected into HeLa cells for 4 h, followed by treatment

with or without berberine. As shown in Fig. 3C, when compared to the control group, the expression levels of EBNA1 were not
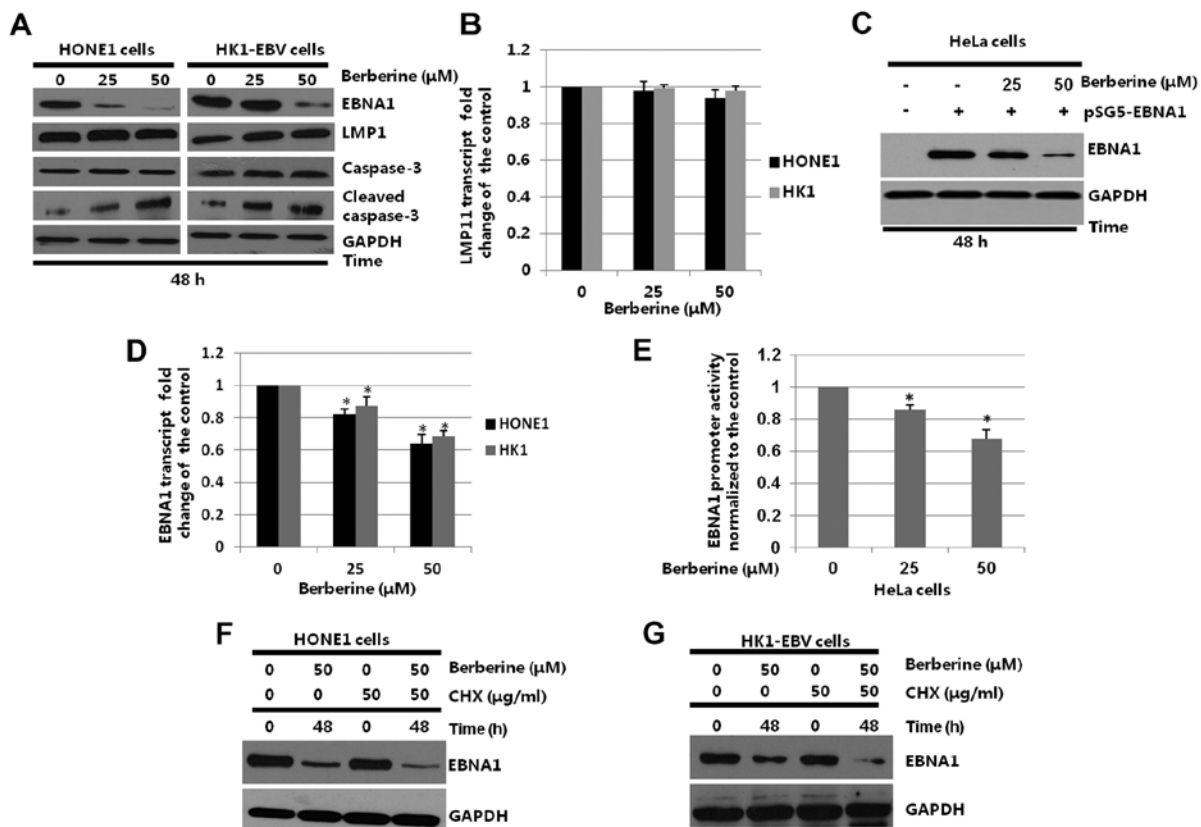
Figure 3. Effect of berberine on the transcription, expression and half-life of the EBNA1 protein in HONE1 or HK1-EBV cells. (A) HONE1 or HK1-EBV cells were placed in 6-well plates and treated with the vehicle control (0.006% DMSO) or berberine (25 or 50 μM) for 48 h. Whole-cell extracts were prepared and western blotting was performed to analyze the expression of EBNA1, caspase-3, cleaved caspase-3 and GAPDH. (B) HONE1 and HK1-EBV cells were treated with the vehicle control (0.006% DMSO) or berberine (25 or 50 μM) for 24 h. The mRNA levels of LMP1 were detected by quantitative-PCR. The level of LMP1 transcript in cells treated with the vehicle control was set as 1. (C) HeLa cells were transfected with pSG5 or pSG5-EBNA1 as indicated, followed by a 44-h berberine (25 or 50 μM) treatment beginning at 4 h after transfection. Whole-cell extracts were prepared and western blotting was performed to analyze the expression of EBNA1 and GAPDH. (D) HONE1 or HK1-EBV cells were treated with the vehicle control (0.006% DMSO) or berberine (25 or 50 μM) for 48 h. The mRNA levels of EBNA1 were determined by quantitative-PCR. The level of EBNA1 transcript in cells treated with the vehicle control was set as 1; *p<0.05, compared with vehicle control (0.006% DMSO). (E) HeLa cells were co-transfected with pGL3.0-Qp and pRL-TK, followed by a 24 h treatment with the vehicle control (0.006% DMSO) or berberine (25 or 50 μM) beginning at 4 h post-transfection. Cell lysates were harvested and the activity of the promoter was determined by a dual-luciferase reporter assay; *p<0.05, compared with the vehicle control (0.006% DMSO). (F) HONE1 and (G) HK1-EBV cells were treated with the vehicle (0.006% DMSO) or berberine (50 μM) for 48 h in the presence or absence of CHX (50 μg/ml) added to the medium 12 h before cell harvesting. Whole-cell extracts were harvested and subjected to western blotting. EBNA1, Epstein-Barr nuclear antigen 1; EBV, Epstein-Barr virus; DMSO, dimethyl sulfoxide; LMP1, latent membrane protein 1; CHX, cycloheximide.

significantly affected in the 25 μM berberine-treated group, but downregulated to 32.5% in the 50 μM berberine-treated group.

To further determine whether the decreased expression of EBNA1 was due to possible alterations at the transcriptional levels, HONE1 and HK1-EBV cells were treated with the vehicle control (0.006% DMSO) or berberine (25 or 50 μM). After 24 h, the total RNAs were harvested and subjected to quantitative-PCR. As shown in Fig. 3D, berberine decreased EBNA1 mRNA levels to 81.23% in the 25 μM and 63.17% in the 50 μM berberine-treated groups, respectively, when compared with the vehicle control (0.006% DMSO) group in the HONE1 cells. In the HK1-EBV cells, the EBNA1 mRNA levels were decreased to 85.39% (p<0.05) in the 25 μM and 65.73% (p<0.05) in the 50 μM berberine-treated groups, respectively, when compared with the vehicle control (0.006% DMSO) group. These results demonstrated that berberine inhibited the mRNA levels of EBNA1 in HONE1 and HK1-EBV cells.

Berberine inhibits the activity of EBNA1 Qp. EBNA1 transcription is initiated from Qp in EBV-associated NPC cells.

According to our previous studies (42,44), the promoters of latent membrane protein 1 (LMP1) and human telomerase reverse transcriptase (hTERT) were studied in HeLa and 293T cells. To determine whether berberine inhibited the activity of EBNA1 promoter, pGL3.0 vector control or pGL3.0-Qp was co-transfected with plasmid pRL-TK into HeLa cells. The HeLa cells were treated with the control (0.006% DMSO) or berberine (25 or 50 μM) for 24 h, at 4 h post-transfection. The whole protein was harvested and the luciferase activity was assessed using the GLO-MAX 20/20 system (Promega). Compared to the control group, the activity of Qp was decreased to 83.4% (p<0.05) and 62.7% (p<0.05) in the 25 and 50 μM berberine-treated groups, respectively (Fig. 3E). The results suggest that the transcriptional activity of EBNA1 was decreased by berberine in the HeLa cell line.

Berberine decreases the half-life of EBNA1. To determine whether berberine decreases the protein half-life of EBNA1, HONE1 and HK1-EBV cells were treated with the vehicle control or berberine, in the presence of CHX. As shown in
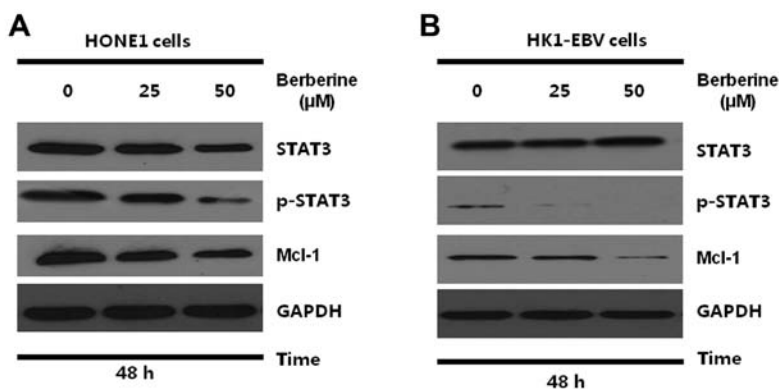
Figure 4. Berberine inhibits the STAT3 signaling pathway in NPC cells. (A) HONE1 and (B) HK1-EBV cells were treated with the vehicle control (0.006% DMSO) or berberine (25 or 50 μM) for 48 h, respectively. Whole-cells extracts were collected and western blotting was performed to analyze the expression of STAT3, p-STAT3 and GAPDH, respectively. STAT3, signal transducer and activator of transcription 3; NPC, nasopharyngeal carcinoma; DMSO, dimethyl sulfoxide.

Fig. 3F and G, berberine decreased the half-life of the EBNA1 protein in the CHX-treated HONE1 and HK1-EBV cells when compared to the vehicle control. The results suggest that berberine decreases the half-life of EBNA1.

Berberine downregulates STAT3 signaling in the NPC cells. The Qp promoter that drives the expression of EBNA1 in EBV-associated tumors is regulated by the JAK-STAT signaling pathway (22,23). STAT3 is essential for the activation of Qp and raises the possibility that aberrant activation of STAT3 may be an essential factor in EBV-associated diseases (22). Since STAT3 represents a potential target for treatment of EBV-associated tumors, it may be beneficial to determine whether the activity of STAT3 is affected by berberine. Therefore, HONE1 and HK1-EBV cells were treated with the vehicle control (0.006% DMSO) or berberine (25 or 50 μM). After 48 h, the total proteins were collected and subjected to western blotting. As shown in Fig. 4A and B, the expression of STAT3 and p-STAT3 were detected in the HONE1 and HK1-EBV cells. Berberine suppressed the expression of p-STAT3 effectively in the HONE1 and HK1-EBV cells. Furthermore, the present study demonstrated that the downregulation of p-STAT3 was associated with the suppressed expression of Mcl-1, which is a downstream survival protein of STAT3. These results suggest that downregulation of EBNA1 by berberine is possibly related to the inhibition of the STAT3 signaling pathway in NPC cells.

Overexpression of EBNA1 attenuates the effect of berberine. To determine whether the berberine-induced decrease in cell viability (Fig. 1B and C) is mainly caused by the inhibited expression of EBNA1, pSG5-EBNA1 or the vehicle control (pSG5) were transfected into HONE1 and HK1-EBV cells. The cells were treated with the vehicle control (0.006% DMSO) or berberine (50 μM) at 4 h post-transfection. After 44 h, the total proteins were harvested and subjected to western blotting. As shown in Fig. 5A and B, the expression levels of EBNA1 were increased by 301.3% in the HONE1 cells, and 368.1% in the HK1-EBV cells, respectively, by transient transfection.

Cell viability was detected after 24 and 48 h. As shown in Fig. 5C and D, the cell viability of both HONE1 and HK1-EBV cells was significantly decreased by berberine.

Following the overexpression of EBNA1, the cell viability was increased by 19.8% (p<0.05) in the HONE1 cells and 22.4% (p<0.05) in the HK1-EBV cells after 24 h, respectively, when compared with the control group (berberine-treated control group). After 48 h, compared to the control group (berberine-treated control group), the cell viability was increased by 24.5% (p<0.05) in the HONE1 cells and 27.6% (p<0.05) in the HK1-EBV cells. Overexpression of EBNA1 increased cell viability and decreased the inhibitory effect of berberine. These results suggest that berberine inhibits the proliferation of EBV-positive HONE1 and HK1-EBV cells possibly through a mechanism related to the decreased expression of EBNA1.

Berberine decreases latent and lytic replication of EBV in EBV-positive NPC cells. To determine whether berberine inhibits lytic replication of EBV, HONE1 and HK1-EBV cells uninduced or induced by TPA and SB were treated with the indicated concentrations of berberine for 48 h. Aciclovir (ACV) was used as a positive control, efficiently decreasing virion production of EBV. After induction, the culture media of HONE1 and HK1-EBV cells were prepared after 48 h incubation and processed for the detection of the EBV EBNA1 fragment. Quantitative-PCR and western blotting were used to detect the effect of berberine on lytic replication of EBV. As shown in Fig. 6A and B, berberine decreased viral genome copies in both uninduced and induced HONE1 and HK1-EBV cells. As expected, ACV, which was used as a positive control, significantly decreased the viral genome copies in the induced HONE1 and HK1-EBV cells. The results indicated that virions were decreased by berberine in a dose-dependent manner. The aforementioned cell lysates were harvested. Western blotting was used to analyze the effect of berberine on the expression of EBV transcriptional activator BZLF1 encoded by the EBV immediate-early (IE) gene in the HONE1 and HK1-EBV cells. As shown in Fig. 6C and D, the expression levels of the EBV transcriptional activator BZLF1 in the HONE1 and HK1-EBV cells were decreased by berberine in both the uninduced and induced HONE1 and HK1-EBV cells. The results suggest that berberine decreased latent and lytic replication of EBV in the HONE1 and HK1-EBV cells.
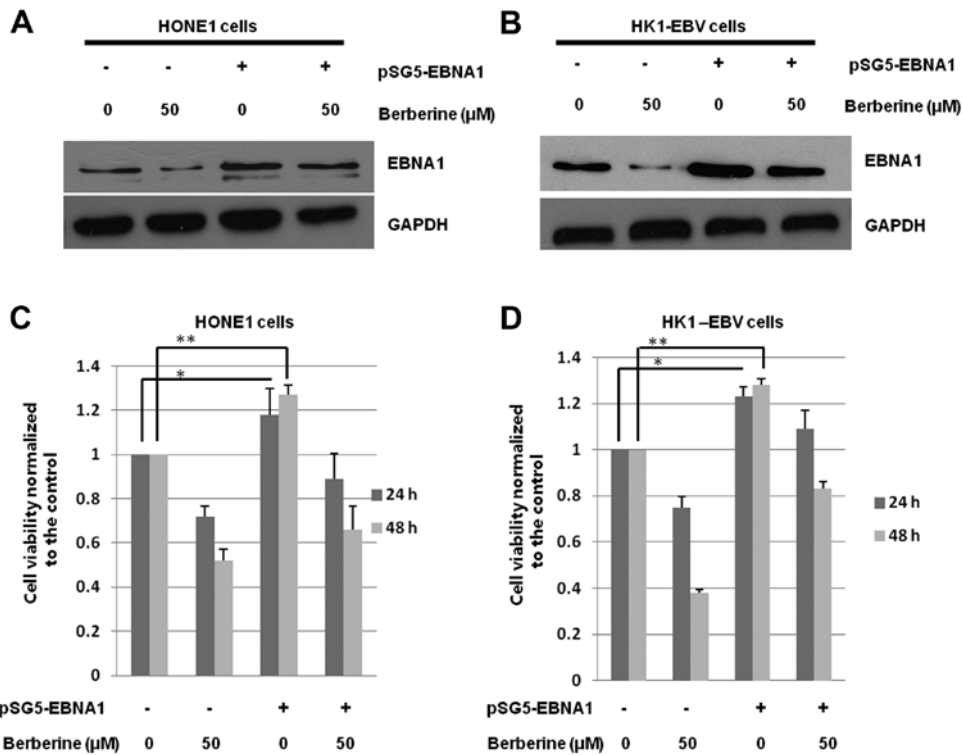
Figure 5. Overexpression of EBNA1 attenuates the inhibitory effect of berberine on the cell viability of EBV-positive NPC cells. (A) HONE1 and (B) HK1-EBV cells were transiently transfected with empty vector pSG5 ('-' means pSG5) or pSG5-EBNA1 ('+' means pSG5-EBNA1), and then, treated with berberine (50 μM) at 4 h post-transfection. After 44 h, total proteins were harvested and western blotting was performed to determine the EBNA1 and GAPDH expression levels. The cell viability of (C) HONE1 and (D) HK1-EBV cells was determined by CCK-8 assay after 24 or 48 h, respectively; *p<0.05, **p<0.01 compared with the control group. EBNA1, Epstein-Barr nuclear antigen 1; EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma.
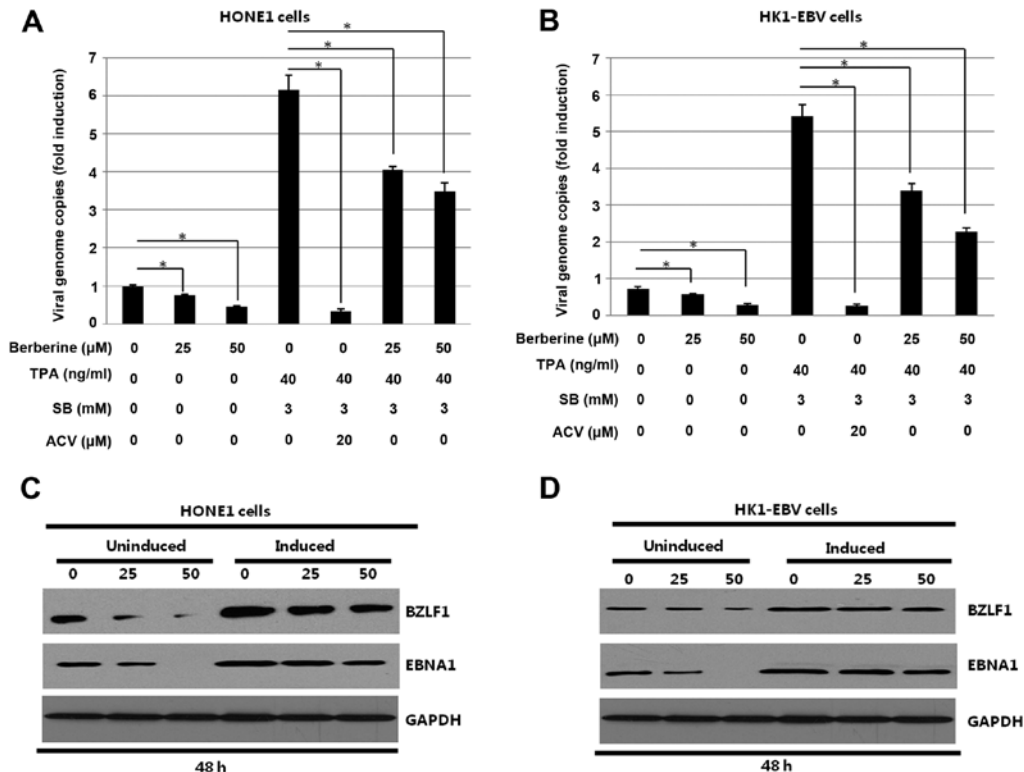

Figure 6. Berberine inhibits EBV virion production in EBV-positive NPC cells. (A) HONE1 and (B) HK1-EBV cells were treated with TPA (40 ng/ml) and SB (3 mM) for EBV induction. After 3 h, uninduced and induced HONE1 and HK1-EBV cells were treated with the vehicle control (0.006% DMSO) or indicated concentrations of berberine for 48 h. Subsequently, the culture medium was harvested for quantitative-PCR analysis with EBNA1 primers. Each sample was normalized to the amount of the GAPDH gene; *p<0.05, compared with the control group. The aforementioned (C) HONE1 and (D) HK1-EBV cell lysates were harvested and subjected to western blotting. EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SB, sodium butyrate; DMSO, dimethyl sulfoxide.
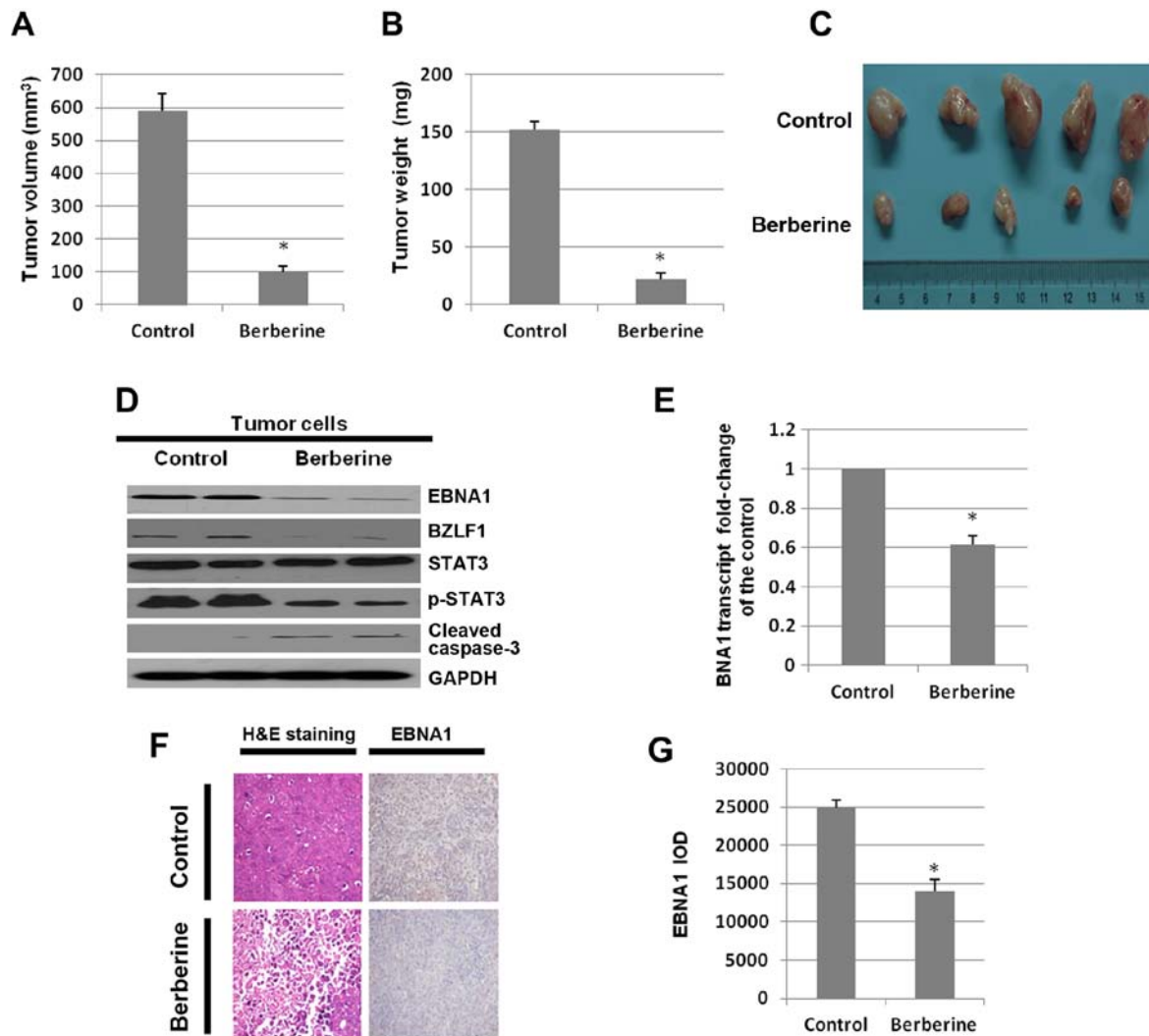
Figure 7. Treatment of NOD/SCID mice with berberine inhibits the growth of NPC *in vivo*. NOD/SCID mice with hypodermic tumors were treated with DMSO or berberine (10 mg/kg, three times a week) for three weeks totally. (A) Tumor volumes and (B) tumor weights were evaluated at the end of the experiment. Whole values are expressed as the means ± SD of five mice; *p<0.05, compared with the control group (DMSO only). (C) Representative images of tumors from the vehicle control (DMSO) and the berberine-treated group are shown. (D) Tumor tissues were collected and subjected to protein extraction, followed by western blotting with the indicated antibodies. Two of the three independent experiments are shown. (E) Total RNAs of tumor tissues were extracted. The EBNA1 mRNA levels in the tumor tissues were detected by quantitative-PCR. The expression of EBNA1 mRNA was normalized against GAPDH levels; *p<0.05, compared with the control group (DMSO only). (F) Tumor tissues were subjected to H&E staining. Immunohistochemical staining using anti-EBNA1 was used to detect EBV-positive cells in tumor tissues. Randomly selected micrographs are presented. (G) The EBV-positive cells from the immunohisto-chemical staining experiments with anti-EBNA1 were quantified and displayed; *p<0.05, compared with the control group (DMSO). NPC, nasopahryngeal carcinoma; EBNA1, Epstein-Barr nuclear antigen 1; EBV, Epstein-Barr virus; IOD, integrated option density.

Berberine inhibits tumor growth in severely immunodeficient mice. Since the aforementioned results demonstrate the inhibition of NPC cell proliferation by berberine, the possible effects of berberine *in vivo* were investigated. To determine whether berberine inhibits the growth of the EBV-positive NPC in NOD/SCID mice, 1×10^7 HONE1 cells were subcutaneously inoculated in the flanks of the mice. After seven days, a dose of 10 mg/kg of berberine or DMSO was administered via subcutaneous injection three times a week. After three weeks, the mice were euthanized and the volumes and weights of the tumors were assessed. The tumor volumes of the DMSO-treated group were larger than that of the berberine-treated group (98.75±13 vs. 590.5±12.3 mm³, n=5, p<0.05; Fig. 7A). Mice treated with berberine had a significantly lower tumor weight (22.25±7.5 vs. 152.5±10.2 mg, n=5, p<0.05; Fig. 7B). Images

of tumors in mice treated with DMSO or berberine are shown in Fig. 7C.

To determine the mechanism by which berberine inhibits tumor growth, the expression levels of EBNA1, BZLF1, p-STAT3 and cleaved caspase-3 in the tumor tissues were analyzed by western blotting. Compared to the DMSO-treated group, berberine downregulated the expression level of EBNA1 significantly and induced tumor cell apoptosis via the caspase-3 pathway. In addition, the expression of BZLF1 was decreased by berberine (Fig. 7D). Then, total RNAs were extracted from tumor tissues. The mRNA levels were analyzed by quantitative-PCR. The mRNA level of EBNA1 in the berberine-treated group was decreased to 61.3% of the control group (Fig. 7E).

Both the vehicle control (DMSO) and the berberine-treated tumor tissue groups were analyzed by H&E staining and

EBNA1 immunostaining (Fig. 7F). The tumor tissues of the berberine-treated group were found to have necrosis and a small amount of inflammatory cell infiltration. The number of EBNA1-positive cells in the berberine-treated mice was significantly decreased compared to the control group. As shown in Fig. 7G, the number of EBNA1-positive cells in the tumor tissues of the berberine-treated group were decreased by 40.2% ($p < 0.05$) compared to those of the DMSO-treated group. The results suggest that berberine significantly inhibits the growth of EBV-positive NPC cells *in vivo*.

Discussion

Nasopharyngeal carcinoma (NPC) is distinguished from other types of head and neck cancers due to its unique sensitivity to radiotherapy and chemotherapy (49). Although a number of drugs have been used as chemotherapy agents (such as cisplatin and cetuximab), a relapse rate as high as 34% still confounds clinicians (50). Therefore, novel therapeutic strategies such as molecular-targeted therapy are needed. EBNA1 has been shown to alter the cellular environment and contribute to cell survival, proliferation, immortalization and tumorigenesis, providing an attractive target for Epstein-Barr virus (EBV)-associated diseases (51). Our previous study demonstrated that Hsp90 inhibitors block outgrowth of EBV-infected malignant cells through an EBNA1-dependent mechanism (41). Thus, development of new drugs that can inhibit the activity of EBNA1 may be therapeutically useful. In the present study, we investigated the effect of berberine on EBV-positive NPC cells *in vitro* and *in vivo*. Our results suggest that berberine effectively inhibits EBV-positive NPC cell proliferation, and induces cycle arrest and apoptosis.

Berberine, an active ingredient extracted from the herb *Coptidis rhizome*, which has long been used as an anti-diarrheal, a stomachic, an antibiotic, and an anti-inflammatory agent in China has been reported to have anticancer properties in multiple cancer cell lines, such as breast, colon and liver cancer (30,31,52). EBNA1 is reported to be an essential protein in all NPC tumors, and is the only EBV protein needed for the persistence and replication of EBV episomes (53). Our findings revealed that berberine decreased EBNA1 expression in two different EBV-positive NPC cell lines and EBV-negative HeLa cells transfected with pSG5-EBNA1 transiently-expressing EBNA1. The activity of the EBNA1 promoter in EBV latency II infection was inhibited by berberine in HeLa cells. In addition, the stability or half-life of EBNA1 was decreased by berberine. These results suggest that berberine inhibited the expression EBNA1 at both the mRNA and protein levels in HONE1 and HK1-EBV cells.

Our previous study demonstrated that triptolide suppressed the activity of LMP1 promoter ED-L1 in latency III type EBV-positive B lymphocytes (42). In NPC, EBV exhibits a type II latency program. In the present study, the mRNA level of EBNA1 was decreased by berberine. Moreover, the activity of the EBNA1 promoter Qp was inhibited by berberine, suggesting that inhibition of EBNA1 promoter activity resulted in the inhibition of EBNA1 at the transcriptional level.

Previous studies have shown that STAT3 is an attractive target for anticancer therapy (24). STAT3 is an important transcriptional factor that functions in numerous physiological processes including cancer development, inflammation, immunity and wound healing. In human breast (MDA-MB-231) and pancreatic (Panc-1) cancer cell lines, the STAT3 inhibitor suppressed the viability, survival, growth and malignant transformation, effectively (54). STAT3 also contributes to EBV and KSHV-mediated cancers by promoting cell proliferation, angiogenesis and invasion. In addition, recent evidence indicated that aberrant activation of STAT3 is essential for the tumorigenesis and progression of NPC (55). STAT3 was activated almost immediately by EBV upon infection of primary cells. EBV utilizes STAT3 to inhibit cellular self-protection functions. A previous study implicated STAT3 as a biologically relevant STAT for the activation of Qp and L1-TR promoters and raises the possibility that aberrant activation of STAT3 may be a contributing factor to EBV-associated tumorigenesis (23). Furthermore, evidence has demonstrated that aberrant activation of the STAT3 pathway is associated with tumor invasion and metastasis in various types of cancer. STAT3 is constitutively activated without any stimulus in HONE1 and HK1-EBV cells. In the present study, the expression levels of p-STAT3 and Mcl-1 (a downstream survival protein of STAT3) were significantly downregulated by berberine. Our results indicate that downregulation of the expression levels of EBNA1 in NPC may be at least partially a result from the inhibition of STAT3 activity by berberine. The mechanism in which STAT3 mediates berberine-induced inhibition of EBNA1 may be studied in the future.

In addition to LMP1, EBNA1 contributes to viral transcription and multiple effects on cellular proteins and pathways that promote cell survival and proliferation (56-58). Our laboratory recently demonstrated that overexpression of LMP1 increases the cell viability of EBV-positive B lymphocytes, B95-8 and P3HR cells (42). In the present study, we found that overexpression of EBNA1 increased the cell viability of HONE1 and HK1-EBV cells. The results demonstrated that berberine decreased the proliferation and activity of EBV-positive NPC cells partly due to the decrease in EBNA1 expression.

In addition to the latent infection of EBV, the lytic reactivation of EBV has been reported to be strongly associated with several human diseases, including NPC. Inhibition of the EBV lytic cycle has been shown to be of great benefit in the treatment of EBV-associated diseases. Consistent with the effect of berberine in decreasing the expression of EBNA1 in EBV-latent infected cells, the present study demonstrated that berberine decreased the expression of the lytic gene BZLF1 in uninduced and induced EBV-positive NPC cells. At present, the mechanism involved in the inhibition of gene expression and lytic replication of EBV by berberine is not very clear and warrants further study. Collectively, berberine induces cell cycle arrest and apoptosis, downregulates the expression of EBNA1 and suppresses lytic replication of EBV in EBV-positive NPC cells.

In addition to the inhibitory effect of berberine on EBV-positive NPC cells *in vitro*, we also investigated the therapeutic effect of berberine on NPC in a xenograft mouse model. Consistent with the ability of Hsp90 inhibitors to suppress tumors in NOD/SCID mice by decreasing the expression of EBNA1 (41), berberine displayed a significant effect on the inhibition of tumor growth in NOD/SCID mice induced by the injection of HONE1 cells. Moreover, berberine

decreased the expression of EBNA1 and BZLF1 in the xenograft tumor cells.

In the present study, our results demonstrated the ability of berberine to decrease cell proliferation, induce cell cycle arrest and promote apoptosis in the EBV-positive NPC cells. The inhibitory activity of STAT3 provides evidence justifying the decrease of EBNA1 levels induced by berberine. In addition, berberine has the ability to inhibit the lytic replication of EBV-positive NPC cells. Thus, berberine may be used as a novel therapy in the treatment of EBV-associated tumors including NPC.

Acknowledgements

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