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 expression 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
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 caicaealensis* (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 downregulation of the expression of various tumor-related proteins including HER2, Bcl-2, COX-2 and EGFR, and the upregulation of the expression of various tumor-related proteins of human breast cancer cells (MCF-7) through the downregulation of the expression of various tumor-related proteins including HER2, Bcl-2, COX-2 and EGFR, and the upregulation of the expression of various tumor-related proteins.

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**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% CO₂. Berberine chloride, cycloheximide (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 EcoRI-XbaI 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'-CGCAAGCTTTCGTCAGGTCCTG-3'. 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'-CGCAAGCTTTGTAAGGATAGCATG-3' (YouBio). The amplified sequence was digested with BglII and HindIII (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 1x10⁴ 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 microimmunoanalyser (BioTek Instruments, Inc., Winooski, VT, USA).

**Flow cytometric analysis.** Apoptosis was quantified using an Annexin V-FITC/propidium iodide (PI) apoptosis detection
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 (25 and 50 µM, respectively) for 24 h, 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.

EBV copy number analysis. HONE1 and HK1-EBV cells were transfected 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 detection system 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, 2.5 mM MgCl₂, 20 µl, pH 8.0) was added to inhibit the activity of DNase I. 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 phenylmethylsulfonyl fluoride (PMSF) and 0.5% cocktail protease inhibitor (Roche) using a micro-scaper. 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% BBP, 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.

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 CaCl₂, 2.5 mM MgCl₂) 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 protease K (0.1 mg/ml) [sample:protease 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 protease 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'-GTGTGGACGAGCTGGAGAGAA-3' and reverse, 5'-GTTGAGACCCGGATGTG-3' (YouBio). The quantitative-PCR conditions were as follows: a 5-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).

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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. As shown in Fig. 1D, treatment with a series of increasing concentrations of berberine (25, 50, and 100 µM) for 24 and 48 h, respectively. Similar results were also found 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).

**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 decreased from ~10 to 90% after berberine treatment for 24 and 48 h. The 50% inhibitory concentration (IC50) 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 IC50 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
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
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) 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.

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 3, the protein levels were assessed using western blotting.

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 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 lysate 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.

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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 down-regulation 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.

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 to 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. EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma; TPA, 12-0-tetradecanoylphorbol-13-acetate; SB, sodium butyrate; DMSO, dimethyl sulfoxide.
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, $1\times 10^7$ 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 measured. The tumor volumes of the DMSO-treated group were larger than that of the berberine-treated group ($98.75\pm 13$ vs. $590.5\pm 12.3$ mm$^3$, n=5, p<0.05; Fig. 7A). Mice treated with berberine had a significantly lower tumor weight ($22.25\pm 7.5$ vs. $152.5\pm 10.2$ 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 immunohistochemical staining experiments with anti-EBNA1 were quantified and displayed; *p<0.05, compared with the control group (DMSO). NPC, nasopharyngeal carcinoma; EBNA1, Epstein-Barr nuclear antigen 1; EBV, Epstein-Barr virus; IOD, integrated option density.
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.

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