Inhibition of migration and invasion by berberine via inactivation of PI3K/Akt and p38 in human retinoblastoma cell line

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Abstract

Background. As a clinically important natural isoquinoline alkaloid, berberine has been reported to possess various pharmacological effects.

Objectives. This study was aimed to investigate the effect of berberine on cell migration and invasion in human retinoblastoma (Rb) cells.

Material and methods. The cytotoxicity of berberine was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After being stimulated with berberine under various concentrations, the cell migration and invasion were evaluated by transwell assay. Then, the expression levels of epithelial-mesenchymal transition (EMT) markers were determined by quantitative reverse transcription PCR (qRT-PCR) and western blot analysis. Furthermore, the phosphorylation levels of protein kinase B (Akt) and p38 were detected by western blot analysis. Finally, the effect of phosphatidylinositol-3-kinase (PI3K) and p38 inhibitors on cell migration and invasion was estimated by transwell assay. Untreated cells acted as control for all the experiments.

Results. The concentrations of berberine for further studies were controlled in a range of 0 to 100 μM. The cell migration and invasion were both suppressed by berberine in a dose-dependent manner compared to the control ($p < 0.05$ or $p < 0.001$). Berberine remarkably down-regulated expression of E-cadherin and up-regulated expression of vimentin and α-SMA compared to the control ($p < 0.01$ or $p < 0.001$). Furthermore, the phosphorylation levels of Akt and p38 were both down-regulated by berberine in comparison to the control. Furthermore, the addition of berberine accompanied by LY294002 or SB203580 significantly suppressed cell migration and invasion compared to the addition of berberine alone ($p < 0.05$).

Conclusions. Berberine suppressed cell migration and invasion via inactivation of PI3K/Akt and p38.

Key words: berberine, invasion, epithelial-mesenchymal transition, phosphatidylinositol 3-kinase/protein kinase B/Akt, p38

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Introduction

Retinoblastoma (Rb) is the most common eye tumor which arises in the retina and represents 2.5–4% of pediatric cancers.[1] The etiology of Rb is the mutation of the Rb tumor-suppressor gene.[2] As a cancer that occurs most often in children before the age of 5 years, Rb affects 2–5 per million children in the USA and Europe.[3] The most frequent clinical manifestations of Rb are leukocoria (white reflection in the pupil) and strabismus (macular involvement).[4] Substantial therapies have been reported to control Rb, such as enucleation, focal therapy, gene therapy, cryotherapy, transpupillary thermotherapy, chemotherapy, and radiotherapy.[5–8] The therapies vary depending on the size of the tumor. Despite the good prognosis of Rb, mortality induced by development of a 2nd tumor remains high. Therefore, novel therapy should be further investigated.

Berberine is an isoquinoline alkaloid (Fig. 1A) isolated from the traditional Chinese medicine Coptis.[9] Extensive research within the past decade proposes that berberine possesses a wide range of pharmacological activities, including anti-diabetic, anti-inflammatory, and anti-tumoral activity.[10–13] Moreover, berberine has been reported to inhibit human colon cancer cell migration via down-regulation of integrin.[14] Chou et al. have implied that berberine could induce cytotoxicity in breast cancer cells.[15] Yip et al. illustrated that berberine induced apoptosis in liver cancer cells.[16] A recent study suggests that berberine is a promising safe anti-cancer agent through affecting the mitochondria.[17] To our knowledge, there is little study focus on the effect of berberine on Rb. Thus, we are interested to know whether berberine can play an efficient role against Rb.

The cell migration and invasion of cancer cells allow them to change position and make the neoplastic cells enter lymphatic and blood vessels for dissemination. After that, metastatic growth occurs in distant organs.[18] As a consequence, the control of cancer cell migration and invasion is a substantial focus of cancer treatment. The present study was designed to investigate the effects of berberine on cell migration, invasion and epithelial-mesenchymal transition (EMT) in human Rb Y-79 cells, which would make a contribution for the discovery of novel therapeutic methods. A previous study has demonstrated that phosphatidylinositol 3-kinase (PI3K)/Akt and p38 are involved in cell migration and invasion, thus we employed specific PI3K inhibitor 2-(4-morpholinyl)-8-phenyl-chromone (LY294002) and p38 inhibitor SB203580 to thoroughly investigate the underlying mechanism.[19]

Material and methods

Cell culture

The human Rb Y-79 cell line was purchased from American Type Culture Collection (ATCC, Manassas, USA). Cells were cultured in RPMI-1640 medium (Gbico, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 U/mL streptomycin (Gbico, Rockville, USA). Cell cultures were maintained in an incubator with a humidified atmosphere of 5% CO_2 at 37°C.

Berberine preparation

Berberine (purity ≥95%, Sigma, St. Louis, USA) was dissolved in dimethyl sulfoxide (DMSO) and filtrated by 0.2 μm disc filters (Millipore, Billerica, USA), resulting in appropriate amounts of stock solution (100 mM in DMSO). The stock solution of berberine was diluted with cultured medium to achieve the indicated concentrations. The final concentration of DMSO was less than 0.2%.

Cell viability assay

To estimate the cytotoxicity of berberine, the cell viability of Rb cells was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in line with the standard method described above.[20] Briefly, Rb cells were seeded onto a 96-well plate (Corning, Corning, USA) with a density of 4×10^3 cells/mL for 24 h. After that, the cells were treated with berberine under various concentrations (0, 5, 10, 20, 40, 80 and 160 μM) for 24 h or 48 h. Following removal of the medium and cell washing by phosphate buffer solution (PBS), 10 μL of MTT solution (5 mg/mL, Sigma, St. Louis, USA) was added to each well accompanied by incubation at 37°C for 4 h. Then, 200 μL of DMSO was added to solubilize the formazan. After measurement by a microplate reader (Bio-Rad, Hercules, USA) at 570 nm, the cell viability was calculated by comparison with untreated cells.

Migration and invasion assays

Cell migration and invasion assays were both performed with Hanging Cell Culture-inserts (BD Biosciences, San Jose, USA) with pores of 8.0 μm in 24-well plates (Corning, Corning, USA). Retinoblastoma cells were firstly stimulated with berberine under various concentrations and maintained for 24 h. After that, cells were treated with trypsin and resuspended with a density of 1×10^5/mL in serum-free medium. When the lower chamber was filled with medium containing 10% FBS, the cells were placed in the upper chamber. After culturing for 24 h, the non-migrated cells which stayed at the surface of the membrane were gently removed with a cotton-tipped swab. Cells that migrated across the filter as well as attached to the underside of the membrane were fixed with methanol and stained with 0.1% crystal violet. Finally, the stained cells were collected and counted under a light microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Whether the inserted filter was pre-coated or not was the difference between the migration assay and invasion assay. In terms of the

invasion assay, the filter was pre-coated with 200 μg/mL Matrigel (BD Biosciences, San Jose, USA). As for the migration assay, the filter was not coated with Matrigel.

Quantitative reverse transcription of polymerase chain reaction

According to the corresponding manufacturer's instructions, isolation of total RNA, reverse transcription of RNA and PCR reactions were performed with TRIzol (Invitrogen, Carlsbad, USA), GoScript™ Reverse Transcription System (Promega, Madison, USA) and Power SYBR Green PCR master mix (Applied Biosystems, Foster City, USA), respectively. Primers were designed and synthetized as shown in Table 1 (Sangon Biotech, Shanghai, China). The results were estimated with $2^{-\Delta\Delta Ct}$ method and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for mRNAs.[21]

Table 1. Primers used in this study

Gene	Primer sequence
E-cadherin	forward 5′- TTCTGCTGCTCTTGCTGTTT -3′ reverse 5′- TGGCTCAAGTCAAAGTCCTG -3′
Vimentin	forward 5′- GCCCTTAAAGGAACCAATGA -3′ reverse 5′- AGCTTCAACGGCAAAGTTCT -3′
α-SMA	forward 5′- AGGTAACGAGTCAGAGCTTTGGC -3′ reverse 5′- CTCTCTGTCCACCTTCCAGCAG -3′
GAPDH	forward 5′- CATCAATGGAAATCCCATCA -3′ reverse 5′- TTCTCCATGGTGGTGAAGAC -3′

α-SMA - α-smooth muscle actin; GAPDH – glyceraldehyde 3-phosphate dehydrogenase.

Western blot analysis

After lysing by RIPA buffer (Beyotime, Shanghai, China) and centrifuged at 4°C, the protein in lysate was quantified using bicinchoninic acid (BCA) assay kit (Pierce, Rockford, USA). Fifty micrograms of protein was loaded and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the protein was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, USA) and blocked with 5% skim milk (Nestlé, Shuangcheng, China) in Tris-buffered saline with Tween 20 (TBST). Subsequently, the membranes carrying blots were incubated with primary antibodies against E-cadherin (ab1416), vimentin (ab8978), α-smooth muscle actin (α-SMA, ab5694), β-actin (ab8226) (all from Abcam, Cambridge, UK), phosphorylated Akt (p-Akt, Ser473, 4060) and phosphorylated p38 (p-p38, 4511) (both from Cell Signaling, Beverly, USA) at 4°C overnight. After rinsing with TBST, the PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at 37°C for 1 h. Protein bands were visualized utilizing the EasyBlot enhanced chemiluminescence (ECL) kit (Sangon, Shanghai, China) and LAS-3000 (Fuji Photo Film, Tokyo, Japan). The housekeeping gene was β-actin.

Statistical analysis

All experiments were repeated 3 times. The results were presented as the mean ±standard deviation (SD) or the mean + SD. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, San Diego, USA). The p-values were calculated using the one-way analysis of variance (ANOVA) or Student's t-test. Significant differences were distinguished with $p < 0.05$.

Results

Cytotoxicity of berberine on retinoblastoma cells

Berberine is one kind of natural isoquinolyl-alkaloid which exist in a large number of medicinal materials. The molecular structure of berberine is shown in Fig. 1A. In order to examine the effect of berberine on cell viability of Rb cells, we employed MTT assay to analyze the cytotoxicity of berberine to Rb cells. In terms of berberine stimulation for 24 h or 48 h, concentrations ranging from 0 to 100 μM were suitable for further investigation (Fig. 1B). When the concentration of berberine was higher than 100 μM, the cell viability was too low to conduct other experiments. Thus, the concentrations of berberine for further studies were controlled in a range of 0 to 100 μM.

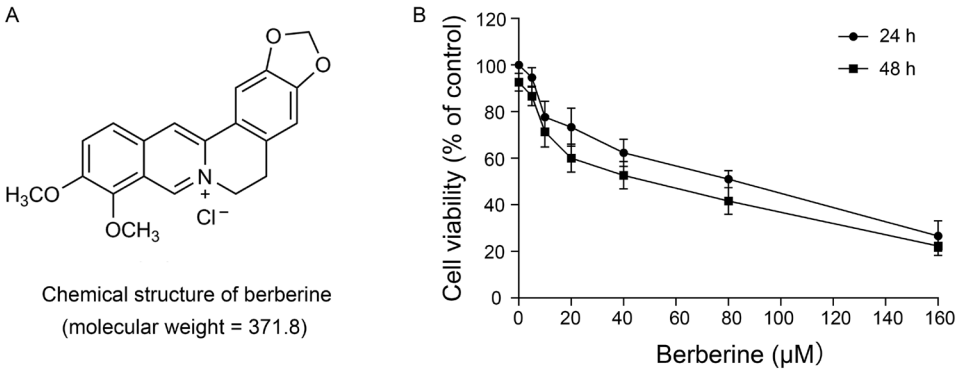

Fig. 1. The effect of berberine on the cell viability in human Rb cells

A – chemical structure of berberine; B – retinobladtoma cells were treated with berberine under various concentrations (0, 20, 40, 60, 80, 100, 120, 140 and 160 μM) for 24 h or 48 h. Thereafter, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The data presented was the mean of 3 independent experiments. Error bars indicate standard deviation (SD).

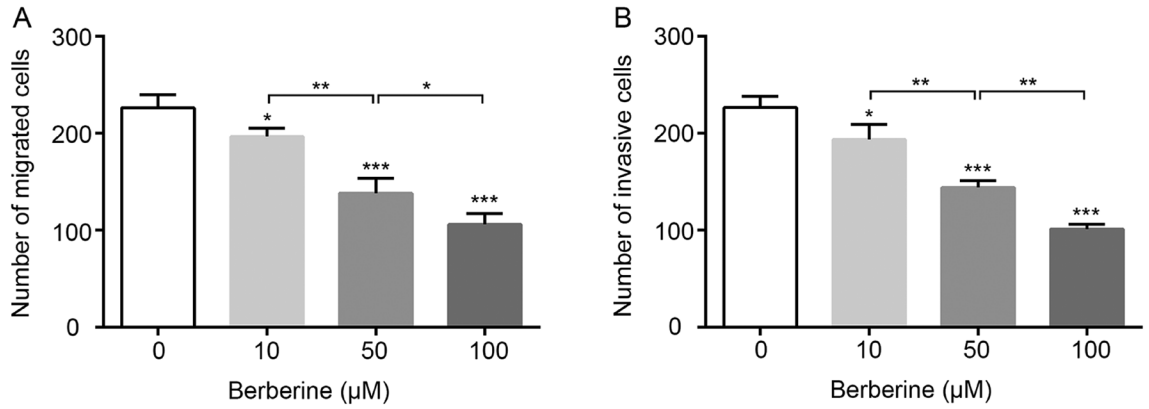

Fig. 2. The suppressive effect of berberine on cell migration and invasion in human Rb cells

Retinoblastoma cells were stimulated by berberine under 0, 10, 50 or 100 μM. After stimulation for 24 h, the cell migration (A) and invasion (B) were both measured by transwell analysis. The data presented was the mean of 3 independent experiments. Error bars indicate standard deviation (SD). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The significance marked at the top of the columns refers to comparisons with the group treated with 0 μM berberine.

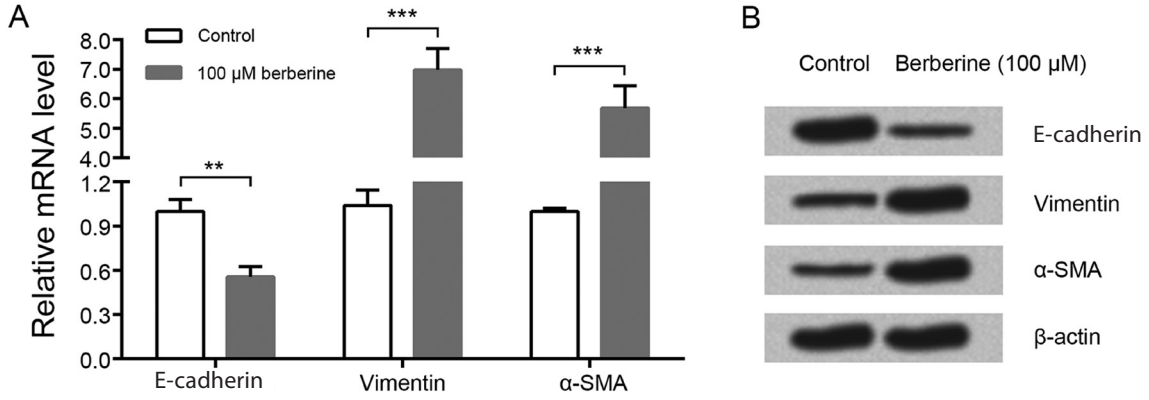

Fig. 3. The inhibitory effect of berberine on epithelial-mesenchymal transition (EMT) in human Rb cells

Retinoblastoma cells were stimulated with or without berberine (100 μM). Cells treated without berberine acted as the control. After stimulation for 24 h, the mRNA (A) and protein (B) levels of EMT markers were respectively estimated by quantitative reverse transcription PCR (qRT-PCR) and western blot analysis. The data presented was the mean of 3 independent experiments. Error bars indicate SD. ** $p < 0.01$; *** $p < 0.001$; α-SMA – α-smooth muscle actin.

Berberine suppressed cell migration and invasion of retinoblastoma cells in a dose-dependent manner

The cell migration and invasion assays were performed with berberine stimulation under various concentrations (0, 10, 50 and 100 μM). The cells without berberine treatment acted as a control group. In Fig. 2A, 2B, the cell migration and invasion of Rb cells were both suppressed by berberine under 10, 50 and 100 μM in comparison with controls ($p < 0.05$ or $p < 0.001$). Further comparison between the cells treated with 50 μM of berberine and 10 μM of berberine ($p < 0.01$) as well as 50 μM of berberine and 100 μM of berberine ($p < 0.05$ or $p < 0.01$) implied that berberine suppressed cell migration and invasion in a dose-dependent manner.

Berberine inhibited epithelial to mesenchymal transition in retinoblastoma cells

In view of the great importance of epithelial to mesenchymal transition (EMT) in the development of invasive cancer cells, we assessed the effect of berberine on EMT markers in Rb cells. The mRNA and protein levels of EMT markers were respectively estimated by qRT-PCR and western blot analysis. Retinoblastoma cells were treated with or without berberine (100 μM) and cells treated without berberine acted as the control. As shown in Fig. 3A, berberine treatment significantly decreased E-cadherin mRNA levels and increased vimentin and α-SMA mRNA levels compared to controls ($p < 0.01$ or $p < 0.001$). The effect of berberine on protein levels of E-cadherin, vimentin and α-SMA was in accordance with mRNA levels (Fig. 3B). Thus, we drew the conclusion that berberine inhibited EMT in Rb cells.

Berberine suppressed cell migration and invasion via inactivation of PI3K/Akt and p38 in retinoblastoma cells

The protein levels of p-Akt and p-p38 were evaluated by western blot analysis. Retinoblastoma cells were treated with or without berberine (100 μM) and the cells treated without berberine acted as the control. In Fig. 4A, the protein levels of p-Akt and p-p38 were both down-regulated by berberine stimulation. Furthermore, Rb cells were stimulated with berberine (100 μM) alone or accompanied with PI3K inhibitor LY294002 (10 μM, Sigma, San Francisco, USA) or p38 inhibitor SB203580 (10 μM, Sigma, St. Louis, USA). Cells treated without berberine acted as the control. In Fig. 4B, 4C, cell migration and invasion were remarkably inhibited by the addition of berberine compared to controls ($p < 0.01$ or $p < 0.001$). Reasonably, co-stimulation of berberine with LY294002 or SB203580 markedly suppressed cell migration and invasion in comparison with stimulation with berberine alone ($p < 0.05$). Taken together, we inferred that berberine suppressed cell migration and invasion through inactivation of PI3K/Akt and p38 in Rb cells.

Discussion

The prognosis of Rb alone is excellent in patients, with a cure rate of 95% in developed countries. Nonetheless, the existing therapies may enhance the risk of occurrence of secondary neoplasms. Hence, the development of novel non-mutagenic therapy is urgent for avoiding a 2nd tumor. In the present study, we found that berberine significantly suppressed cell migration, invasion and EMT. Additionally, the phosphorylation level of Akt and p38 were both down-regulated by stimulation of berberine. Further studies on co-stimulation of berberine with a specific inhibitor of PI3K or p38 implied that berberine suppressed cell migration and invasion via suppression of PI3K/Akt and p38 phosphorylation.

High cell migration and invasion are two substantial characteristics for cancer cells. Numerous reports have illustrated the suppressive effect of berberine on cancer cells. Berberine suppressed migration and invasion of human SCC-4 tongue squamous cancer cells via inhibition of focal adhesion kinase (FAK), nuclear factor-κB (NF-κB), IκB kinase (IKK), urokinase-type plasminogen activator (u-PA), matrix metalloproteinase 2 (MMP-2), and MMP-9.[22] Berberine has also been reported to inhibit the migration and invasion of T24 bladder cancer cells through reducing the expression of heparanase.[23] Moreover, berberine exerted suppressive effect on migration and invasion of hepatocellular carcinoma cells via altering the expression of plasminogen activator inhibitor-1 (PAI-1) and u-PA.[24] The effect of berberine on the cell migration and invasion of Rb cells was consistent with the studies described above.

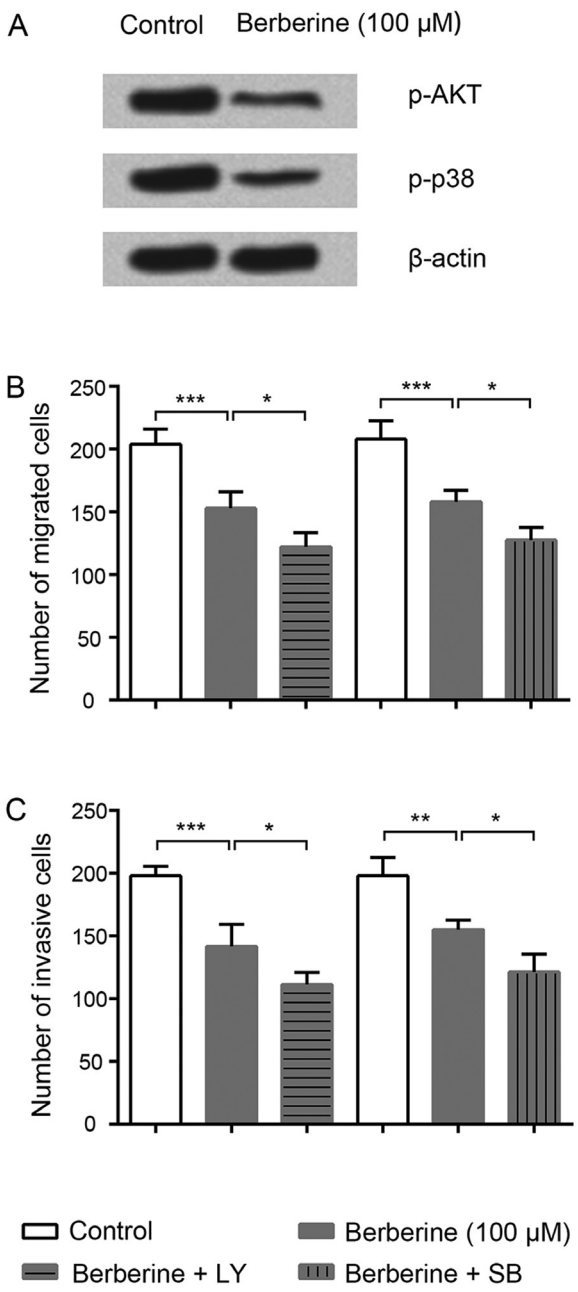

Fig. 4. The relationship between the suppressive effect of berberine on cell migration and invasion and inactivation of PI3K/Akt and p38

Cells were stimulated with or without berberine (100 μM). After stimulation for 24 h, the protein levels of p-Akt and p-p38 were estimated by western blot analysis (A). Furthermore, the cells were stimulated with berberine (100 μM) alone or respectively co-stimulated with 2-(4-morpholinyl)-8-phenyl-chromone (LY294002, 10 μM) or SB203580 (10 μM). After stimulation for 24 h, the cell migration (B) and invasion (C) were evaluated by transwell analysis. Cells treated without berberine acted as the control. The data presented was the mean of 3 independent experiments. Error bars indicate SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; LY – LY294002; SB – SB203580.

The complex metastasis of cancer consists of 5 continuous steps – proliferation, migration, invasion, adhesion, and angiogenesis.[25] Epithelial to mesenchymal transition is characterized by the loss of cell-cell adhesion, which makes cells gain mesenchymal features and stem-like

properties. The obvious effect of EMT on cells is improved migratory capacity and invasiveness, and reduced cell adhesion as well as resistance to cancer treatment.[26] A previous study claimed that inhibition of the mTOR pathway attenuated the migration and invasion of gallbladder cancer via EMT inhibition.[27] Recently, microRNA-133a was reported to suppress the migration and invasion of esophageal cancer cells by targeting the EMT regulator SOX4.[28] In the present study, the results relevant to EMT markers suggested that berberine markedly inhibited EMT, which might be one reason for the suppressive effect of berberine on cell migration and invasion of Rb cells.

Protein Kinase B is a kind of serine-threonine kinase which acts as the primary downstream mediator of PI3K and participates in a large numbers of bioprocesses.[29] P38 is one of the mitogen activated protein kinases (MAPKs), which also has been reported to be involved in various cell processes.[30] A previous study has claimed that a basic helix-loop-helix (bHLH) transcription factor (Twist), could increase migration and invasion of breast cancer cells via transcriptionally up-regulating Akt2.[31] Heparanase has been reported to promote human gastric cancer cells migration and invasion by increasing phosphorylation level of p38.[32] Specifically, Liu et al. have illustrated that inactivation of Akt or p38 induced down-regulation of both matrix metalloproteinase 2 (MMP-2) and MMP-9, leading to suppressive migration and invasion of human glioblastoma cells.[33] In the present study, berberine stimulation significantly decreased protein expression levels of p-Akt and p-p38, implying that the inactivation of Akt and p38 might be another reason for the suppressive migration and invasion of Rb cells. Additionally, the comparison between cells treated with berberine alone and cells treated with berberine accompanied by the extra addition of PI3K inhibitor LY294002 or p38 inhibitor SB203580 seems to verify the inference. Furthermore, extensive investigations have suggested that EMT was induced by Akt or p38 signaling pathway.[34–36] In a word, berberine suppressed cell migration and invasion through inactivation of PI3K/Akt and p38.

In summary, we are providing the first report for the suppressive effect of berberine on cell migration and invasion of human Rb cells in a dose-dependent manner. Furthermore, EMT was markedly inhibited by berberine. In addition, the suppressive effect of berberine on cell migration and invasion was due to the inactivation of PI3K/Akt and p38. Therefore, berberine may represent a new anticancer drug that can prevent metastasis of Rb and become a potential therapeutic agent.

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