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Berberine suppressed epithelial mesenchymal transition through cross-talk regulation of PI3K/AKT and RARα/RARβ in melanoma cells

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ABSTRACT

Berberine is a natural compound extracted from Coptidis rhizoma, and accumulating proof has shown its potent anti-tumor properties with diverse action on melanoma cells, including inhibiting cancer viability, blocking cell cycle and migration. However, the mechanisms of berberine have not been fully clarified. In this study, we identified that berberine reduced the migration and invasion capacities of B16 cells, and notably altered pluripotency of epithelial to mesenchymal transition associated factors. We found that berberine also downregulation the expression level of p-PI3K, p-AKT and retinoic acid receptor α (RARα) and upregulation the expression level of retinoic acid receptor β and γ (RARβ and RARγ). These effects of PI3 kinase inhibitor LY294002 treatment mimicked Berberine treatment except the expression level of RARγ. Moreover, Western blot analysis showed that the decreased PI3K and AKT phosphorylation, increased the epithelial maker E-cadherin, and upregulation level of RARβ while decreased the mesenchymal markers N-cadherin and downregulation level of RARα by incubation with LY294002 in mouse melanoma B16 cells. In conclusion, Our study reveal that berberine can reverse the epithelial to mesenchymal transition of mouse melanoma B16 cells and may be a useful adjuvant therapeutic agent in the treatment of melanoma through the PI3K/Akt pathway and inactivation PI3K/AKT could regulate RARα/RARβ expression.

1. Introduction

Over 90% of cancer-related deaths that can be contributed to the metastasis [1]. Melanoma is the deadliest form of skin cancer because of its tend to metastasize [2]. It is a relatively rare cancer in China, but causes over 65% of skin cancer-related deaths [3]. Cancer metastasis is a major cause for poor survival and prognosis of melanoma patients. Therefore, decreasing the metastasis of melanoma is one of the important goal in medicine.

The Epithelial-to-mesenchymal transition (EMT) is a critical mechanism in tumor cells metastasis [4]. During the process of EMT, cancer cells gain mesenchymal characteristics and lose their epithelial characteristics to become invasive [5]. Some studies found that PI3K/Akt signaling pathway mediates the process of EMT and has attracted wide attention as a novel target for the prevention and treatment of metastatic tumors [6]. Activated PI3K by tyrosine kinase leads to the oncogene AKT [7] and activated Akt could phosphorylate a variety of substrates, thereby affecting kinds of cellular processes, such as cell cycle, cellular growth, differentiation, apoptosis, angiogenesis and migration [8]. In addition, numbers of studies showed that PI3K/Akt pathway is associated with other signaling pathways, including Ras, NF-κB, TGF-β, and Wnt/β-catenin, to directly or indirectly induce the EMT [9]. Furthermore, previous study showed that All-trans retinoic acid (ATRA) enhanced Akt activation regulation by RARα-Akt interaction and the active form of Akt remarkably reduces expression levels of the tumor suppressors RARβ2 [10]. The PI3K/AKT pathway may be also associated with Retinoid signaling pathway.

Berberine, an isoquinoline alkaloid, can be isolated from all kinds of medicinal herbs including *Coptis chinensis*, *Berberis vulgaris*, *Berberis aristata*, *and Berberis aquifolium*, many of which are used in traditional medicines [11]. Berberine was confirmed to present comprehensive pharmacological actions, such as anti-cancer, anti-inflammatory, and anti-microbial effects [12]. Recently, berberine has been proved to possess anti-cancer activities. For example, berberine was discovered to activate AMP-activated

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protein kinase (AMPK) which inhibited effects on cancer cell migration and metastasis [13]. And berberine declined melanoma cancer cell migration by decreasing the expressions of prostaglandin E2, prostaglandin E2 receptors and cyclooxygenase-2 [2]. Recently, some study showed that in Human Chondrosarcoma, berberine induced G2/M arrest through activated PI3K/AKT cell line [14]. Also, in neuroblastoma cells, berberine induced neuronal differentiation via suppression of cancer stems cell and EMT [15].

However, the effects of berberine on melanoma metastasis and the molecular mechanisms still remain unknown. Therefore, the purpose of this study was to investigate the effect of berberine on metastasis of mouse melanoma B16 cells and the underlying mechanisms. These dates could provide a novel sight of the anti-metastasis mechanisms of berberine.

2. Materials and methods

2.1. Cell lines and reagents

The murine melanoma B16 cells were cultured in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml),10% fetal bovine serum (Hyclone) and incubated at 37 °C with 5% CO_2. Berberine were purchased from Chengdu Mansite Pharmaceutical Co. Ltd. (Chengdu, China). MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was from Sigma (St. Louis, MO, USA). LY294002 was purchased from Calbiochem (San Diego, CA, USA).

2.2. MTT assay

B16 cells were cultured in the 96-well plates for 24 h at a seeding density of 6.0×10^3 cells before treated. After cells treatment with berberine (5 μM, 10 μM, 20 μM, 40 μM, 80 μM, 160 μM) for different times, the medium was removed and 100 μl of MTT reagent (0.5 mg/ml) was added to each well for another 4 h of incubation. At the end of the incubation time, the medium was removed; intracellular formazan was solubilized with 150 μl DMSO and quantified spectrophotometrically (λ = 490 nm) with a microplate reader Victor X3 (PerkinElmer, Waltham, MA, USA).

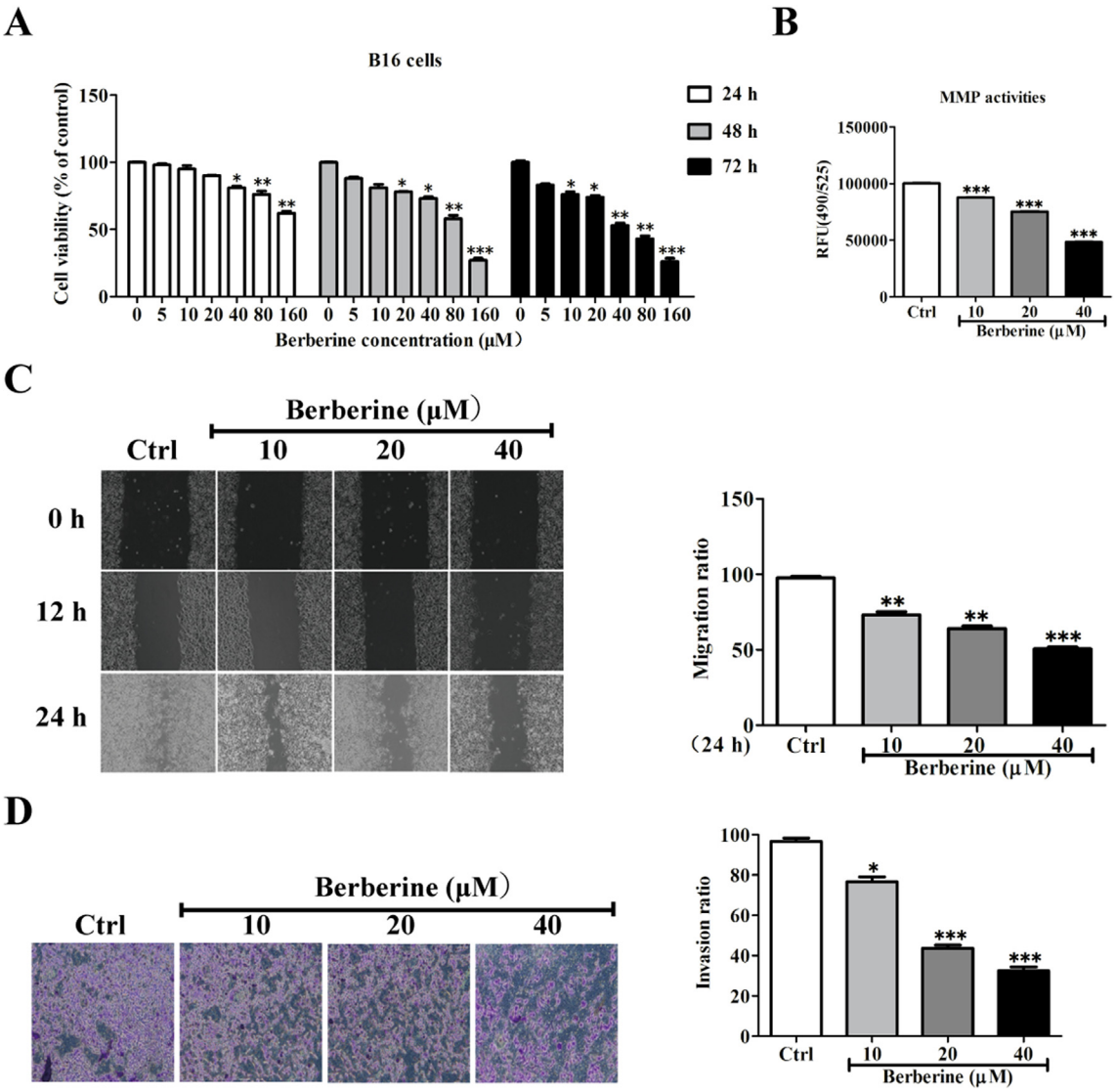

Fig. 1. (A) Cell viability analysis of B16 cells cultured in the presence of BBR for 24, 48 h or 72 h by MTT assay. (B) B16 cells were treated with berberine (10 μM, 20 μM and 40 μM) for 48 h. The culture medium of the cells after treatment were detected the activity of MMPs using AmpliteTM Universal Fluorimetric MMP Activity Assay Kit.(C) Migration of B16 cells treated with BBR (10 μM, 20 μM and 40 μM) and its control was measured by wound-healing.(D) Invasion of B16 cells treated with BBR(10 μM, 20 μM and 40 μM) and its control was measured by transwell.

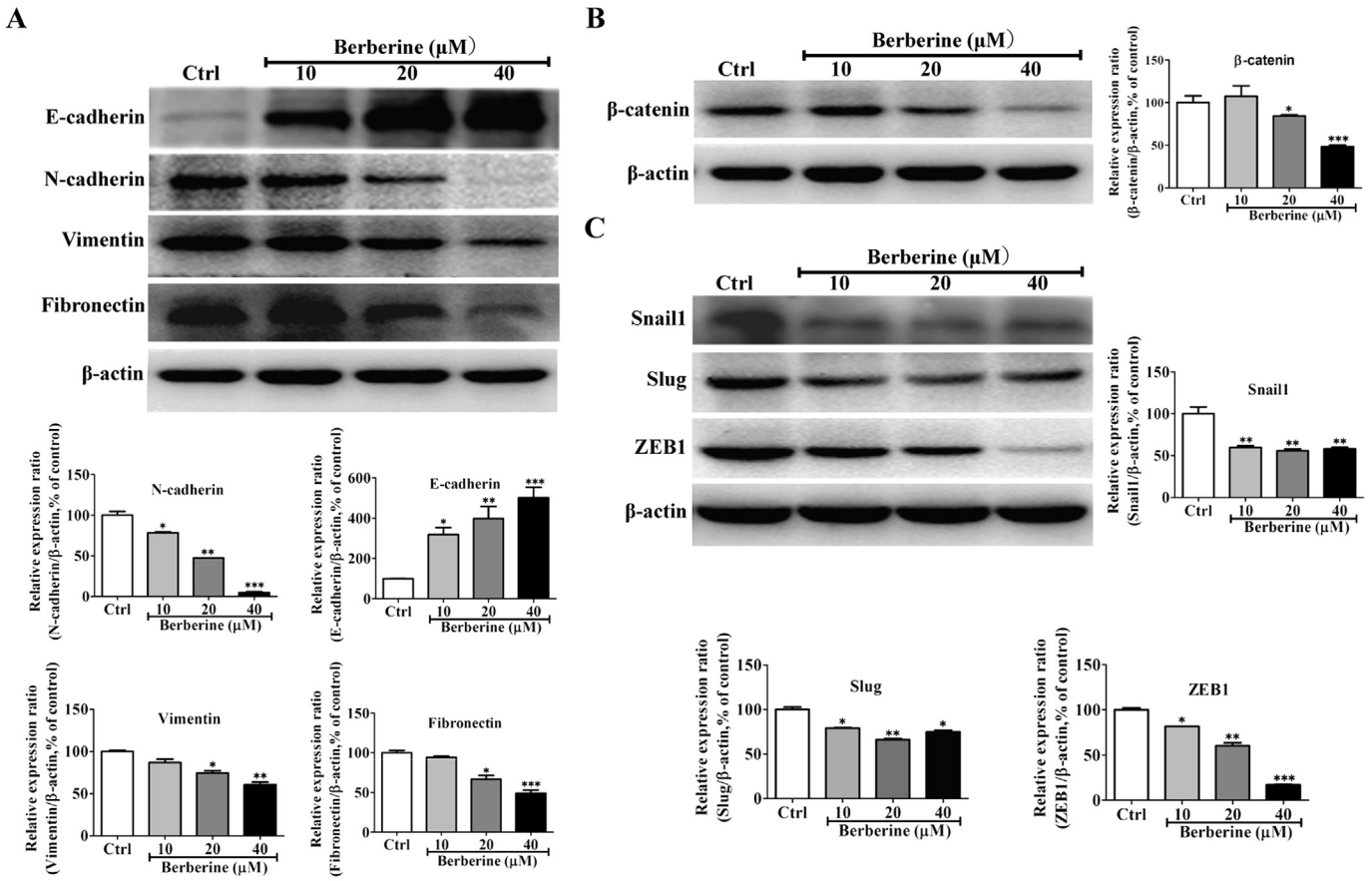

Fig. 2. (A) Expression of EMT markers E-cadherin, N-cadherin, Fibronectin, Vimentin, (B) and β-catenin, (C) and transcription factors Snail1, Slug, ZEB1 in B16 cells treated with BBR(10 μM, 20 μM and 40 μM) and its control were analyzed by Western blotting. The bar graphs (mean ± SD) and representative images are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the B16 group.

2.3. Transwell and wound healing assays

B16 cells (1.5×10^4 cells/well) were cultured in the upper chamber with low-serum medium contained berberine, while high-serum medium (15% FBS) was added to the lower chamber. After 24 h of incubation at 37 °C, cells were fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet for 20 min. Then dissolved the crystal violet in DMSO, and the OD value were detected by Victor X3(PerkinElmer, Waltham, MA, USA) at 600 nm. For wound healing assays, cells were seeded on six-well plates and grown to confluency. Then, the cells were gently scratched with tips to create a mechanical wound and incubated in medium with 0.5% FBS in the absence or presence of berberine for 24 h. Images were taken at 0, 12 h and 24 h using a microscope (Leica, Jena, Germany).

2.4. Western blot analysis

Cells were seeded in 6-well culture plates at a density of 3×10^5 cells/well and treated with berberine (10 μM, 20 μM and 40 μM) and LY294002 (20 μM) for 48 h. RIPA buffer mixed PMSF (RIPA: PMSF = 100:1) was used to harvest and lyse cells. The samples were further incubated with the primary antibodies against β-actin, E-cadherin, N-cadherin, vimentin, fibronectin, β-catenin, snail1, slug, ZEB1, RARα, RARβ, RARγ (Santa Cruz Biotechnology with 1:1000–1:2000 dilutions), PI3K, p-PI3K, AKT, p-AKT, (CST Biotechnology with 1:1000–1:2000 dilutions) followed by HRP (horseradish peroxidase)-labeled secondary. Goat anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology) was used as the secondary antibody. The hybridization signal was examined using enhanced chemiluminescence (ECL). The relative intensities of the blots were quantified by Quantity One (Bio-Rad, Hercules, CA, USA).

2.5. MMP activity assay

The activity of MMPs was detected with AmpliteTM Universal Fluorimetric MMP Activity Assay Kit according to the standard protocols. In brief, 3×10^5 cells were seeded onto 6-well plate and treated with berberine (10 μM, 20 μM, 40 μM) and LY294002 (20 μM) for 48 h. Then 50 μl of supernatants from each well was added to a 96 well plate for detection.

2.6. Statistical analysis

Data were statistical analyzed with SPSS 17.0. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ are determined as significance. Value presented as the means ± standard deviation (SD) by GraphPad Prism software (GraphPad Software, CA, USA).

3. Results

3.1. Berberine suppressed mouse melanoma B16 cells invasion and migration in a dose-dependent manner

The cytotoxic effects of berberine on mouse melanoma B16 cells

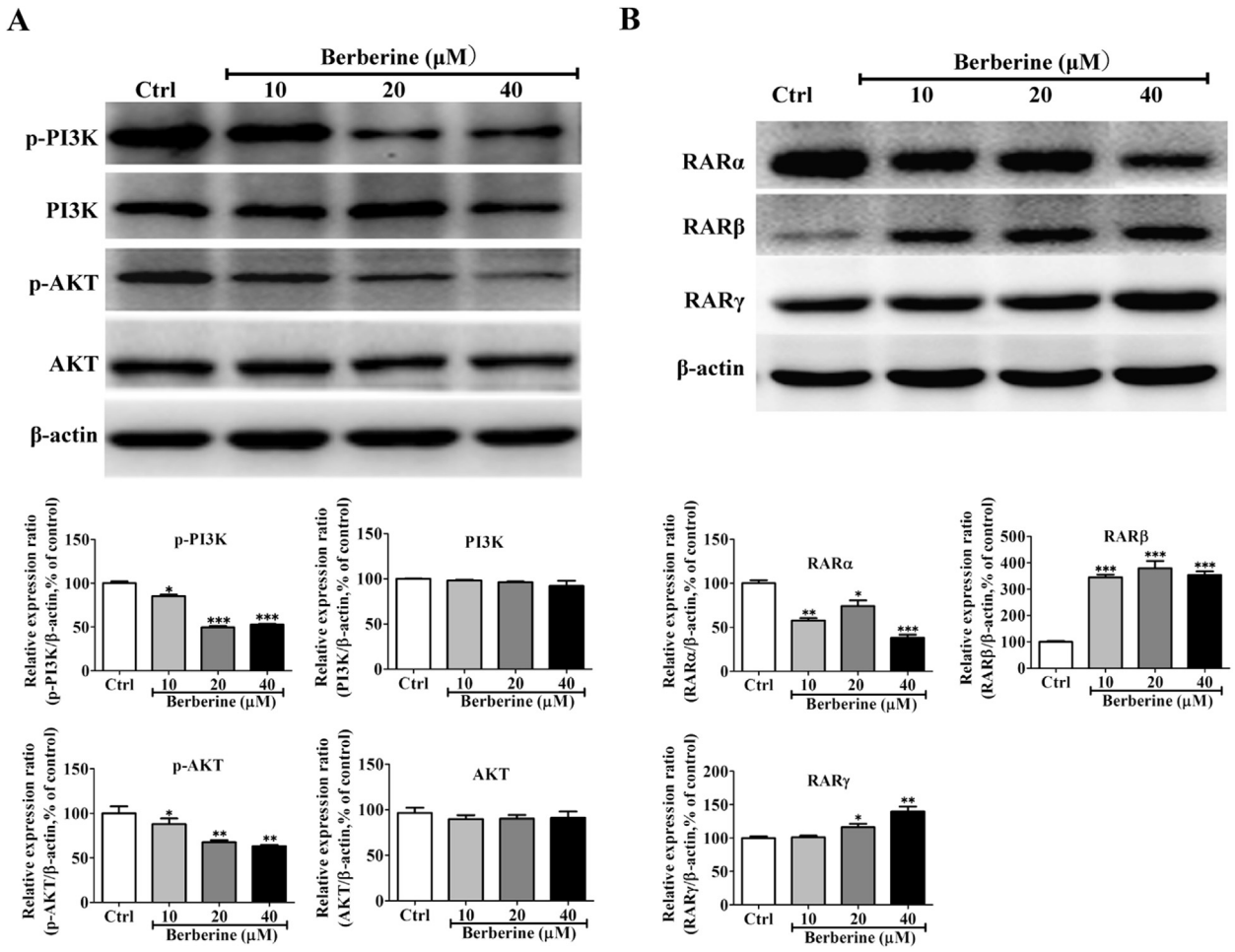

Fig. 3. (A) Phosphorylation and expression of PI3K, phosphorylation and expression of AKT, (B) and the protein levels of RARα, RARβ, and RARγ in B16 cells treated with BBR(10 μM, 20 μM and 40 μM) and its control were analyzed by Western blotting. The bar graphs (mean ± SD) and representative images are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the B16 group.

were first evaluated by MTT cell viability assay. As shown in Fig. 1A, berberine significantly inhibited cell proliferation of B16 cells in both dose- and time-dependent manner. Meanwhile, the MMP activities were markedly suppressed by berberine with reduction in a dose dependent manner (Fig. 1B, $p < 0.001$). Wound healing assay was implemented to confirm if berberine affects cell migration. The result showed that 40 μM berberine suppressed B16 cell migration by 50.5± 1.3% at 24 h (Fig. 1C). Berberine inhibited B16 invasion in a dose-dependent manner, 40 μM of berberine inhibited the invasiveness of B16 cells by 67.53 ± 1.88% (Fig. 1D, $P < 0.001$).

3.2. Berberine significantly inhibited EMT of B16 melanoma cells

To determine whether berberine is associated with EMT, we detected the expression of epithelial and mesenchymal phenotype markers with western blotting. Berberine markedly upregulated epithelial markers, such as E-cadherin. Berberine also significantly declined mesenchymal markers such as N-cadherin, and the expression was downregulated by 95.26± 1.19% when B16 cells were treated with 40 μM berberine (Fig. 2A, $p < 0.01$). Meanwhile, berberine treatment inhibited fibronectin and vimentin expressions. Cadherin-bound β-catenin is needed for cell adhesion. Western blot analysis showed that β-catenin protein levels were decreased by berberine treatment (Fig. 2B). Furthermore, after berberine treatments the expressions of the transcription factors such as Snail1, Slug and ZEB1 were significantly decreased in a dose-dependent manner (Fig. 2C).

3.3. Regulation of the mRNA and protein levels of PI3K/AKT and RARs pathways by berberine

To investigate whether berberine influences PI3K/Akt signaling in melanoma cells, we tested the protein levels of PI3K, its phosphorylation (p-PI3K), Akt and its phosphorylation (p-AKT) in B16 cells in response to treatment with BBR(10 μM, 20 μM, and 40 μM). We found that berberine did not affect the protein levels of total PI3K and AKT, but remarkably inhibited the phosphorylation of the two kinases in a concentration-dependent manner. 40 μM berberine significantly decreased the protein of p-AKT with the decline of 63.12 ± 1.3% (Fig. 3A). In addition, we examined the expression of RARs with western blotting. The result showed that compared to control group, berberine remarkably activated RAR pathway in a dose-dependent manner through increasing the protein expressions of RARβ and RARγ while decreasing that of RARα (Fig. 3 B).

3.4. Berberine inhibited EMT via modulation of PI3K/AKT and RARα/RARβ pathways in B16 cells

To investigate the validity of our results, we detected whether

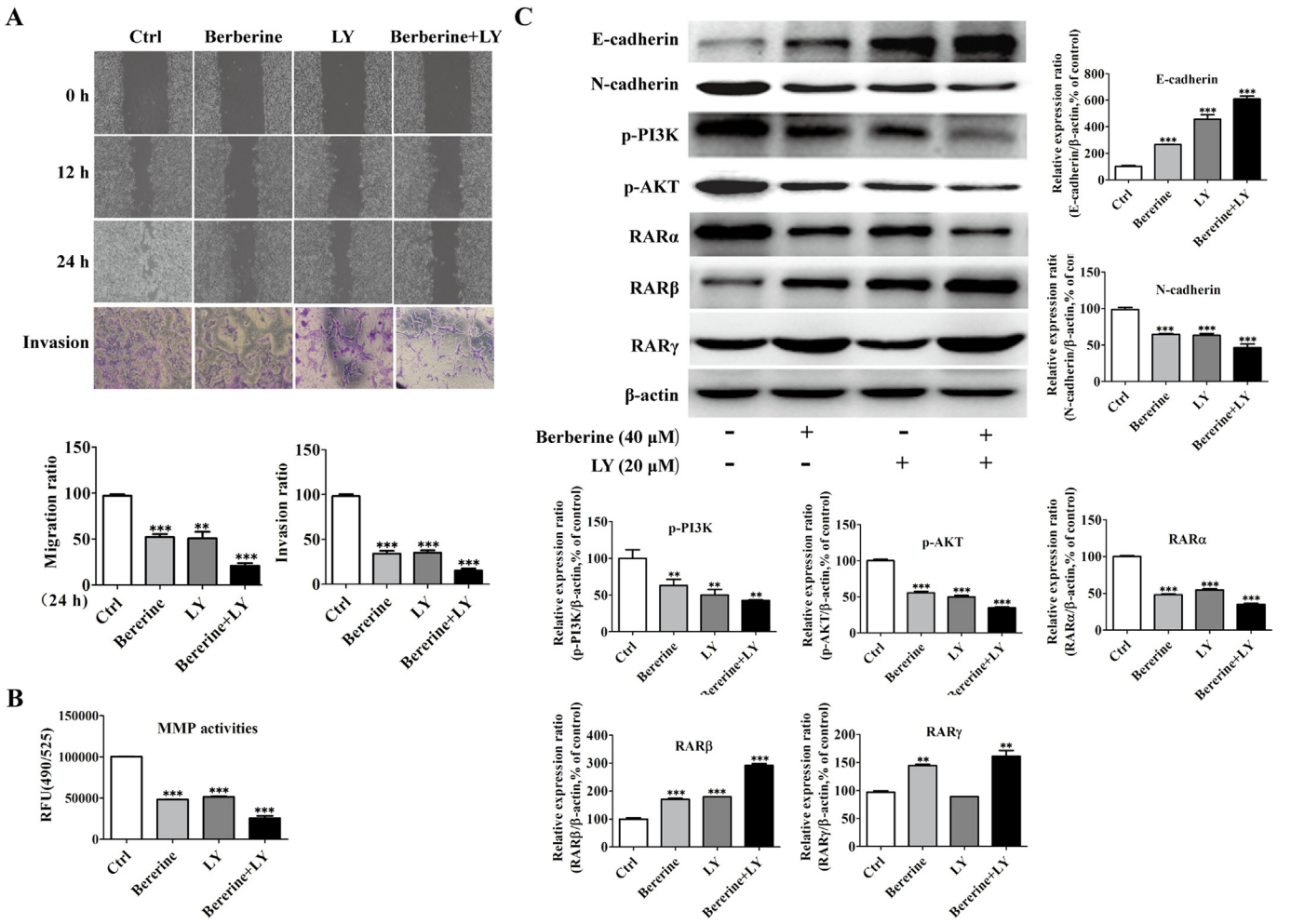

Fig. 4. (A) Migration and Invasion of B16 cells were treated with 20 μM LY294002 (LY, a PI3K inhibitor) alone or in combination with BBR (40 μM) for 24 h and its control was measured by wound-healing and transwell. (B)B16 cells were treated with LY (20 μM) alone or in combination with BBR (40 μM) for 48 h. The culture medium of the cells after treatment were detected the activity of MMPs using AmpliteTM Universal Fluorimetric MMP Activity Assay Kit. (C) B16 cells were treated with LY (20 μM) alone or in combination with BBR (40 μM) for 48 h. Protein expression levels of E-cadherin, N-cadherin, p-PI3K, p-Akt, RARα, RARβ, RARγ were measured by Western blotting. The bar graphs (mean ± SD) and representative images are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the B16 group.

disturbing with the PI3K/Akt pathway by a specific PI3K/Akt inhibitor (LY294002) inhibited the EMT potential of B16 cells. Compared to the control treatment, the migration and the invasion ratio were significantly decreased by LY294002 (20 μM). Accounting for a 50.8 ± 2.82% reduced in the migration and a 64.86 ± 2.71% reduced in the invasion in B16 cells (Fig. 4A, $p < 0.001$). LY29002 treatment also resulted in a remarkable suppression effect on the MMP activities on B16 cells (Fig. 4B). Furthermore, we examined the effect of LY294002 on the levels of proteins including EMT markers, PI3K/AKT and RARs pathway affect by Berberine, as mentioned above. We found that LY294002 notably increased the protein express of E-cadherin, while decreased that of N-cadherin, p-PI3K, p-AKT. In addition, the protein level of RARα was reduced but RARβ was enhanced in cells exposed to LY294002. Then, the RARγ protein level was unchanged. (Fig. 4C). Therefore, LY294002 showed a down-regulation influence that was similar to that of Berberine on B16 cells. These results suggested that berberine suppressed the EMT of highly metastatic B16 melanoma cells via the modulation of the PI3K/Akt and RARα/RARβ (Fig. 4C).

4. Discussion

Cancer cell metastasis is a complex process including migration, invasion, adhesion, and proliferation [16]. Melanoma is the main important cause of death in skin diseases and metastasis in early stage, and metastasis is the primary reasons that cancer therapies against melanoma fail. Therefore, novel metastasis-targeting antitumor drugs are needed to be developed for the migratory potential of melanoma cells. Berberine, as a pharmacological agent, treated a kinds of human diseases has been documented in Asian countries [12] and inhibited cancer metastasis [17]. Previous study found that berberine could decrease the pulmonary metastasis in mice and suppress invasion and migration ability of B16 F-10 melanoma cells through inhibition of MMP [18]. Furthermore, berberine decreased invasion and metastasis ability through COX-2/PGE2 mediated JAK2/STAT3 pathway in colorectal cancer cells [19]. In the current study, we found that berberine suppressed invasion and metastasis of B16 cells and activated some signaling pathways.

Our study found that berberine inhibited cell viability, migration and invasion of B16 cells (Fig. 1A–D). Previous study reported that secreting extracellular proteases such as MMPs family are significant during cancer metastasis [20]. We found that berberine significantly decreased MMP activities (Fig. 1B). EMT, a biologic process found in many types of epithelial cancers including melanoma, is able to increase cancer cells adhesion, invasion and migration [21]. EMT process is initiated by transcription factors

snail1, slug and ZEB1. Of these, transcriptional factor Snail1 binds to the promoter E-box, which suppressed E-cadherin transcription leading to inhibit E-cadherin expression [22]. In addition, during epithelial–mesenchymal transition procession, cancer cells acquire mesenchymal markers, including N-cadherin, vimentin, and fibronectin, followed by tumor metastasis, and proliferation at distant sites [23]. Our data suggested that berberine altered morphological characteristics of cells, which is similar to MET with increased E-cadherin and decreased N-cadherin, vimentin, fibronectin and β-catenin (Fig. 2A–B). Then, berberine inhibited transcriptional factor including snail1, slug, and ZEB1 (Fig. 2C). Our results suggested that berberine reverses epithelial mesenchymal transition and inhibits metastasis in mouse melanoma B16 cells.

The PI3K/AKT pathway is activated in a kinds of oncogenic processes, including cell proliferation, apoptosis, and metastasis [24]. It is well known that PI3K/AKT signaling pathway facilitates EMT signal transition [25]. Moreover, the PI3K/PTEN signaling pathway plays important roles in the cadherin switch by decreasing E-cadherin expression and increasing N-cadherin expression in melanoma cells [26]. In this study, we found that berberine inhibited the protein levels of p-PI3K, and p-AKT when epithelial mesenchymal transition was reversed in mouse melanoma B16 cells. Furthermore, some study showed that over-expression of RARα enhanced Akt phosphorylation in COS-7 cells and interacted with PI3k at the plasma membrane [27]. However, inducing the PI3k/Akt signaling pathway resulted in down-regulation the mRNA and protein levels of RARβ2 [28]. In addition, RARγacts as a tumor suppressor or oncogene in different cancers, depending on the cell-specific context [29]. For example, Gui-Li Huang et al. suggested that RARγ knockdown decreased metastatic abilities in CCA cells [30]. Our study showed that berberine reduced the protein levels of RARα while enhanced the protein levels of RARβ and RARγ (Fig. 3B). PI3K/AKT signaling pathway was closely associated with RARs. Moreover, LY294002, a PI3K/Akt pathway inhibitor, is used to confirm that the suppressive effect of berberine on the EMT process in melanoma B16 cells involves the regulation of the PI3K/Akt pathway and RARα/RARβ proteins (Fig. 4A–C). Therefore we suggested that berberine inactivated PI3K/AKT signaling pathway leading to down-regulation RARα and up-regulation RARβ.

In conclusion, this study suggested that berberine could inhibit cells invasion and migration of mouse melanoma through inhibiting EMT-related protein. The mechanism of the reducing EMT by berberine might be regulation via cross-talk between PI3K/AKT and RARα/RARβ. Furthermore, our study suggests that berberine should be developed as a natural compounds with low-toxicity and low-lost for clinical cancer treatment.

Conflict of interest

All authors declare that no conflicts of interest exist.

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Transparency document

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