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Berberine inhibits the malignant cell phenotype by inactivating PI3K/AKT/mTOR signaling in laryngeal squamous cell carcinoma

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Summary. Background. Berberine is an active compound found in different herbs used in Chinese medicine and is well-known for its potential anticancer properties. The study aimed to figure out the role of berberine in regulating the malignant behavior of laryngeal squamous cell carcinoma (LSCC) cells.

Methods. LSCC cell lines (SNU-899 and AMC-HN-8) were treated with different concentrations of berberine (0-200 μM) to determine its cytotoxicity. The migration, invasion, and apoptosis of LSCC cells were measured by wound healing assays, Transwell assays, and flow cytometry. Western blot was performed for the quantification of proteins involved in PI3K/AKT/mTOR signaling.

Results. The viability of LSCC cells was dose-dependently reduced by berberine. Berberine dampened LSCC cell migration and invasion while augmenting cell apoptosis, as evidenced by a reduced wound closure rate, a decrease in invaded cell number, and a surge in cell apoptosis in the context of berberine stimulation. Importantly, the effects of berberine on the cancer cell process were enhanced by LY294002 (an inhibitor for PI3K) treatment. Moreover, the protein levels of phosphorylated PI3K, AKT, and mTOR were markedly reduced in response to berberine treatment.

Conclusion. Berberine inhibits cell viability, migration, and invasion but augments cell apoptosis by inactivating PI3K/AKT/mTOR signaling in LSCC.

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Introduction

Laryngeal squamous cell carcinoma (LSCC), a common tumor type of head and neck squamous carcinoma, presents high mortality and morbidity (Louw and Claassen, 2008; Fan et al., 2019). Multiple factors including smoking, drinking, and radiation exposure were closely associated with the initiation and development of LSCC (Smith et al., 2019; Song et al., 2020b; Zhang et al., 2020a). In addition, several complications, such as dysphagia, dyspnea, hoarseness, cough, and cervical lymph node metastasis have been observed in the clinic, which poses a great threat to the health and life of LSCC patients (Liu et al., 2022). To date, modern therapies including surgery, chemotherapy, and radiotherapy have been adopted for LSCC treatment (Verro et al., 2023); however, the complicated pathology is a great challenge for this treatment.

Berberine, an isoquinoline alkaloid, is a critical component of *Beberis* species (Cicero and Baggioni, 2016; Habtemariam, 2020). Previous studies reported that berberine possessed several pharmacological functions, such as anti-bacterial, anti-hypertensive, and anti-arrhythmic (Song et al., 2020a; Wang et al., 2017). Furthermore, studies found that berberine attenuated tumor growth in various tumors (Ortiz et al., 2014; Rauf et al., 2021). For instance, berberine downregulated IL-6 to inhibit tumor progression by targeting the JAK2/STAT3 pathways in gastric cancer (Xu et al., 2022). Moreover, berberine suppresses the Hedgehog signaling pathway and cell growth in colorectal cancer

(Sun et al., 2023). Importantly, pretreatment with berberine enhanced the anti-tumoral effects of 5-fluorouracil and cisplatin in LSCC cells (Palmieri et al., 2018). However, the exact role of berberine in LSCC remains unclear.

Mechanistically, berberine was proposed to target several signaling pathways in tumors (Chen et al., 2018; Farooqi et al., 2019; Sun et al., 2022). In detail, berberine induced cytostatic autophagy via inactivating MAPK/mTOR/p70S6K signaling *in vitro* and *in vivo* in human gastric cancer (Zhang et al., 2020b). Moreover, berberine suppresses SCAP/SREBP-1 signaling-induced lipogenesis and cell proliferation in colon cancer (Liu et al., 2020). Furthermore, a combination of berberine and curcumin increased the death of glioblastoma cells by inhibiting the PI3K/AKT/mTOR pathway (Maiti et al., 2019). However, the downstream signaling of berberine in LSCC remains uncharacterized.

In the present exploration, we aimed to clarify the role of berberine in LSCC. Based on published studies, we hypothesized that berberine inhibits the malignant phenotype of LSCC cells by inactivating PI3K/AKT/mTOR signaling. Our research provides a potential and novel strategy for LSCC management.

Materials and methods

Cells

LSCC cell lines (SNU-899 and AMC-HN-8) and human laryngeal epithelial cells (ULA-TVC) were obtained from The Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 units/mL), streptomycin (100 µg/mL), and 2% glutamine (Gibco) with 5% CO_2 at 37°C.

Reagents and treatment

Berberine (purity >98%) and LY294002 (a PI3K inhibitor) were purchased from MedChemExpress (Shanghai, China). Berberine was diluted in Dimethyl Sulphoxide (DMSO, final concentration: 0.1%) according to the manufacturer's instructions and frozen in aliquots at -80°C. Cells were treated with different doses of Berberine and LY294002 (50 µM) for 24 or 48h in different assays.

Cell counting kit-8 (CCK-8) assay

Cells were treated with different doses of berberine and LY294002 (50 µM), and then seeded into the 96-well plate (2×10^4 cells/ml). After incubation for 24 or 48h, 10 µl CCK-8 solution cells were added to each well for 2h incubation at 37°C. Hereafter, the optical density at 450 nm was detected using a microplate reader (BioTek Instruments, Inc.).

Annexin V-FITC/PI double-staining assay

ULA-TVC, AMC-HN-8, and SNU-899 cells (3×10^5 cells/well) were seeded in 6-well plates for 24h incubation. Then, the different doses of berberine and LY294002 (50 µM) were added to each well for 24h. After detaching with 0.05% trypsin, washing with cold phosphate-buffered solution (PBS, Gibco), and centrifuging at 1500 rpm for 5 min at 4°C, cells were gently suspended in the binding buffer and stained with Annexin V-FITC (final dilution: 1 µg/mL) in the dark for 15 min. Subsequently, cells were stained with PI (final dilution: 1 µg/mL) for another 5 min. Finally, the cell apoptosis rate was immediately analyzed by flow cytometry (BD FACSCanto II).

Wound healing assay

ULA-TVC, AMC-HN-8, and SNU-899 cells were digested with trypsin and cultured in 6-well plates (2×10^5 cells/well). Then, cells were treated with berberine (25 or 50 µM) and LY294002 (50 µM). Linear scratches were made on the surface of the cell monolayer with a perpendicular sterile 20 µl pipette tip when the cell confluence reached about 100%. Next, cells were washed with PBS, and scratch-induced cell debris was gently removed. Hereafter, cells were incubated in serum-free medium. After 24h, the scratch width was photographed in a microscope at 0h and 24h, and the migration rate was quantified by ImageJ software.

Transwell assay

The Transwell assay was performed with a Transwell chamber (8 µm; BD Biosciences) precoated with Matrigel (BD Biosciences) to evaluate the invasion of ULA-TVC, AMC-HN-8, and SNU-899 cells. Briefly, cells were treated with berberine (25 or 50 µM) and LY294002 (50 µM), and then placed in the upper chamber containing serum-free medium, and medium containing 10% FBS was added to the lower chamber for 24h incubation. The invaded cells (in the lower chamber) were fixed with 4% formaldehyde, stained with 0.5% crystal violet, and then observed and photographed with an inverted light microscope (Olympus Corporation).

Western blot

RIPA lysis buffer was used to extract proteins from ULA-TVC, AMC-HN-8, and SNU-899 cells. The protein sample was transferred onto PVDF membranes after separation by 10% SDS-polyacrylamide gel electrophoresis. Subsequently, membranes were blocked with 5% skim milk and incubated with primary antibodies at 4°C overnight. After washing with TBST solution three times, the membranes were incubated with

the secondary antibody at room temperature for 1h and developed with chemiluminescence solution. The gray value for protein was determined by ImageJ software. Detailed information on the specific primary antibodies is provided in Table 1.

Statistical analysis

The experimental data are expressed as mean ± standard deviation (SD). Statistical analysis was performed by using GraphPad 7.0 software. Differences between two or multiple groups were analyzed by using the Student's t-test or one-way ANOVA followed by Tukey's *post hoc* test, respectively. $P<0.05$ was considered statistically significant.

Results

Berberine reduces the viability of LSCC cells

First, we evaluated the cell viability of ULA-TVC and LSCC cells treated with berberine for 24 or 48h. As shown in Fig. 1A, berberine does not affect the cell viability of ULA-TVC cells, in contrast, that of SNU-899 and AMC-HN-8 cells is gradually reduced by berberine treatment (25, 50, 100, or 200 μM) for 24h. However, there were no significant differences between the 50, 100, or 200 μM groups. Hence, 25 or 50 μM of berberine was used for the following assay. Moreover, the 48h treatment of berberine induced a great decrease in cell viability (Fig. 1B), which may have an impact on subsequent experimental results. To exclude this effect, berberine was used for 24 h in the following assays.

Berberine facilitates cell apoptosis but dampens LSCC cell migration and invasion

We then further explored the biological role of berberine in LSCC cells. According to flow cytometry analysis, the apoptosis rate of SNU-899 and AMC-HN-8 cells was prominently enhanced by berberine (25 or 50 μM) treatment (Fig. 2A). In addition, the wound healing assay demonstrated that the wound closure rate of SNU-899 and AMC-HN-8 cells was significantly decreased by berberine (25 or 50 μM) treatment (Fig. 2B). Moreover, the Transwell assay revealed that the number of invading SNU-899 and AMC-HN-8 cells was also reduced by berberine (25 or 50 μM) treatment (Fig. 2C). Also, berberine did not affect ULA-TVC cell apoptosis, migration nor invasion of the ULA-TVC cell line (Fig. 3A-C). Taken together, Berberine promotes cell apoptosis but inhibits LSCC cell migration and invasion.

Berberine inactivates PI3K/AKT/mTOR signaling in LSCC cells

Berberine was reported to inactivate PI3K/AKT/mTOR signaling in various tumors, so we hypothesized that berberine exerts a similar mechanism in LSCC. According to western blot analysis, we discovered that phosphorylated PI3K, AKT, and mTOR proteins were upregulated in LSCC cells compared with ULA-TVC cells (Fig. 4A). Moreover, the protein levels of

Table 1. Primary antibody information.

Antibody	Dilution	Number	Source
p-PI3K	1: 1000	#4228	Cell Signaling Technology
PI3K	1: 1000	#4257	Cell Signaling Technology
p-AKT	1: 1000	#4060	Cell Signaling Technology
AKT	1: 1000	#9272	Cell Signaling Technology
p-mTOR	1: 1000	#5536	Cell Signaling Technology
mTOR	1: 1000	#2983	Cell Signaling Technology
β-actin	1: 1000	#4970	Cell Signaling Technology

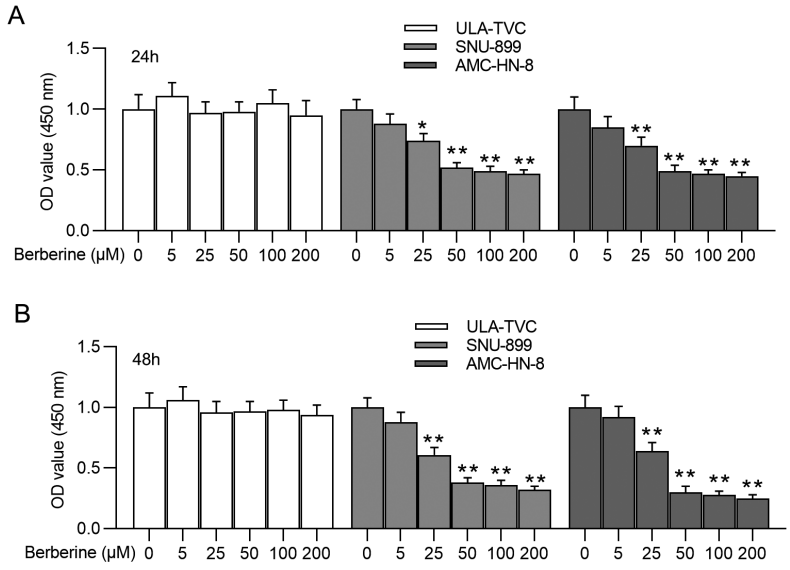

Fig. 1. Berberine reduces the viability of LSCC cells. **A, B.** The cell viability of ULA-TVC, AMC-HN-8, and SNU-899 was evaluated by the CCK-8 assay with different berberine doses for 24h or 48h. *$P<0.05$, **$P<0.01$.

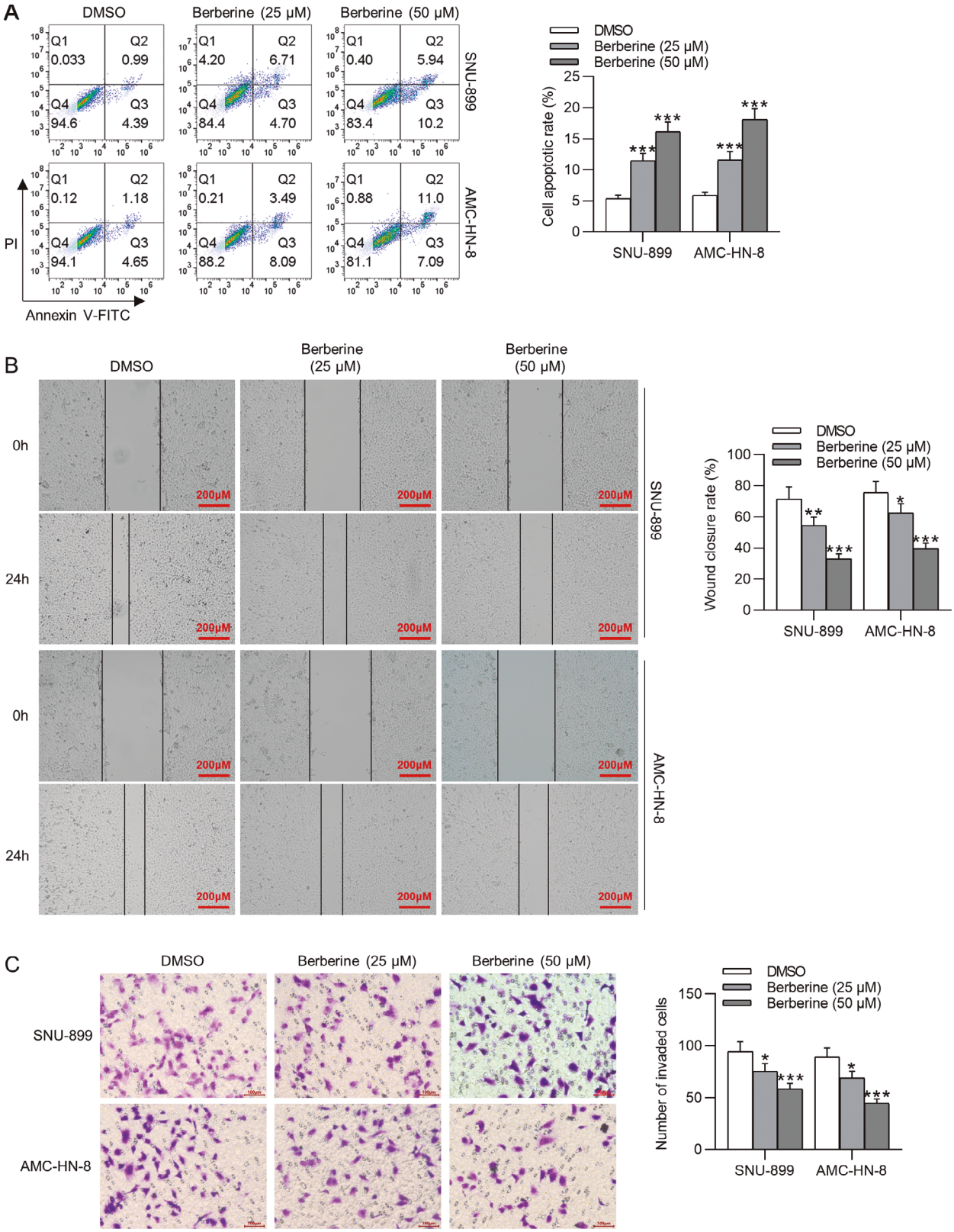

Fig. 2. Berberine facilitates cell apoptosis but dampens LSCC cell migration and invasion. **A.** Cell apoptosis with berberine treatment (25 or 50 μM) was measured by flow cytometry. **B, C.** Cell migration and invasion with berberine treatment (25 or 50 μM) were detected by the wound healing and Transwell assays, respectively. **P*<0.05, ***P*<0.01, ****P*<0.001.

phosphorylated PI3K, AKT, and mTOR were downregulated by berberine (25 or 50 μM) treatment in SNU-899 and AMC-HN-8 cells, while the protein levels of unphosphorylated PI3K, AKT, and mTOR display no significant differences in SNU-899 and AMC-HN-8 cells (Fig. 4B,C). Furthermore, berberine treatment did not affect these proteins in ULA-TVC cells (Fig. 4D). To conclude, berberine inhibits the activation of

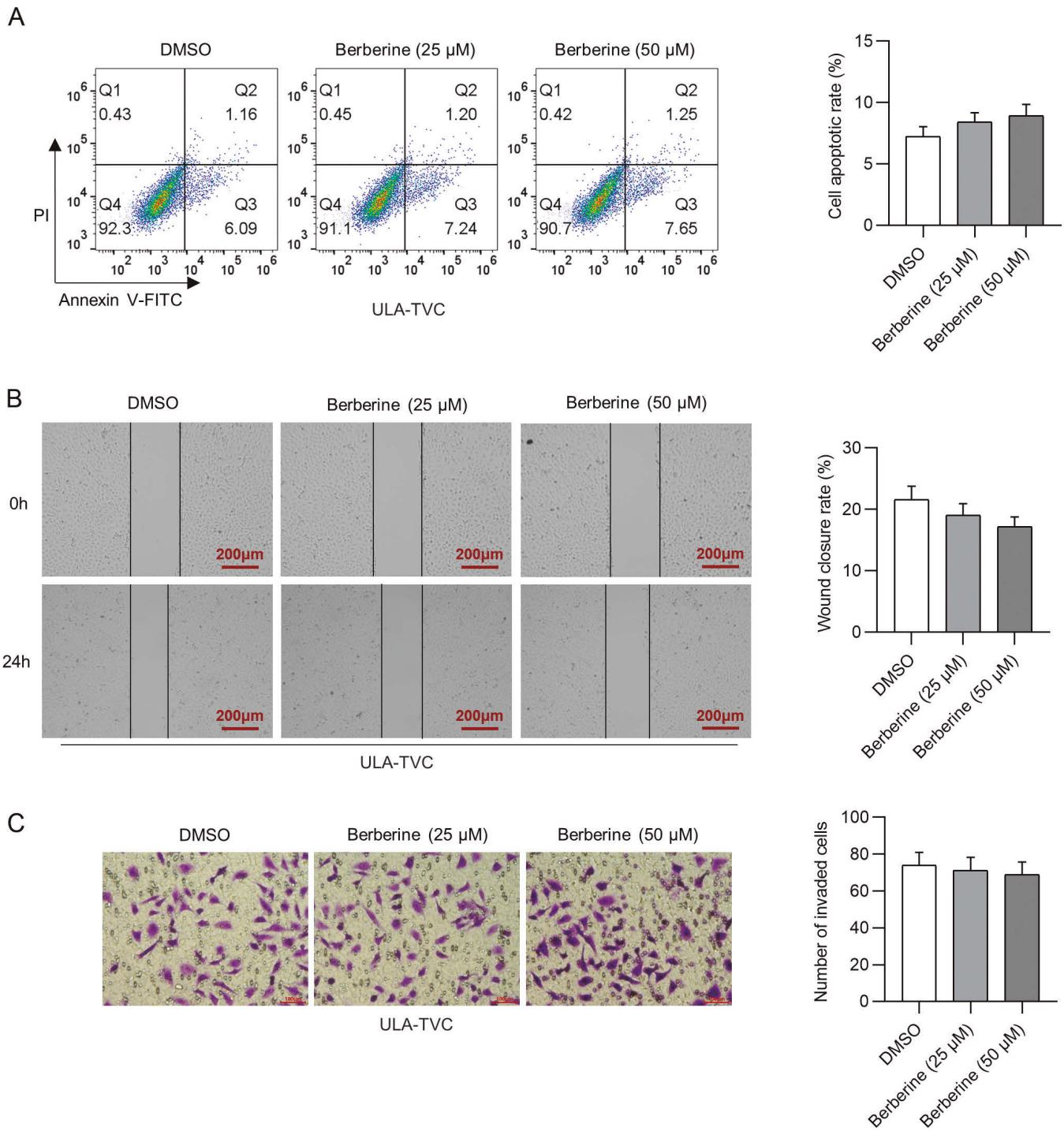

Fig. 3. Berberine did not affect cell phenotype of ULA-TVC cell. Flow cytometry analysis **(A)**, wound healing **(B)**, and Transwell assays **(C)** were performed to evaluate the role of berberine in ULA-TVC cells.

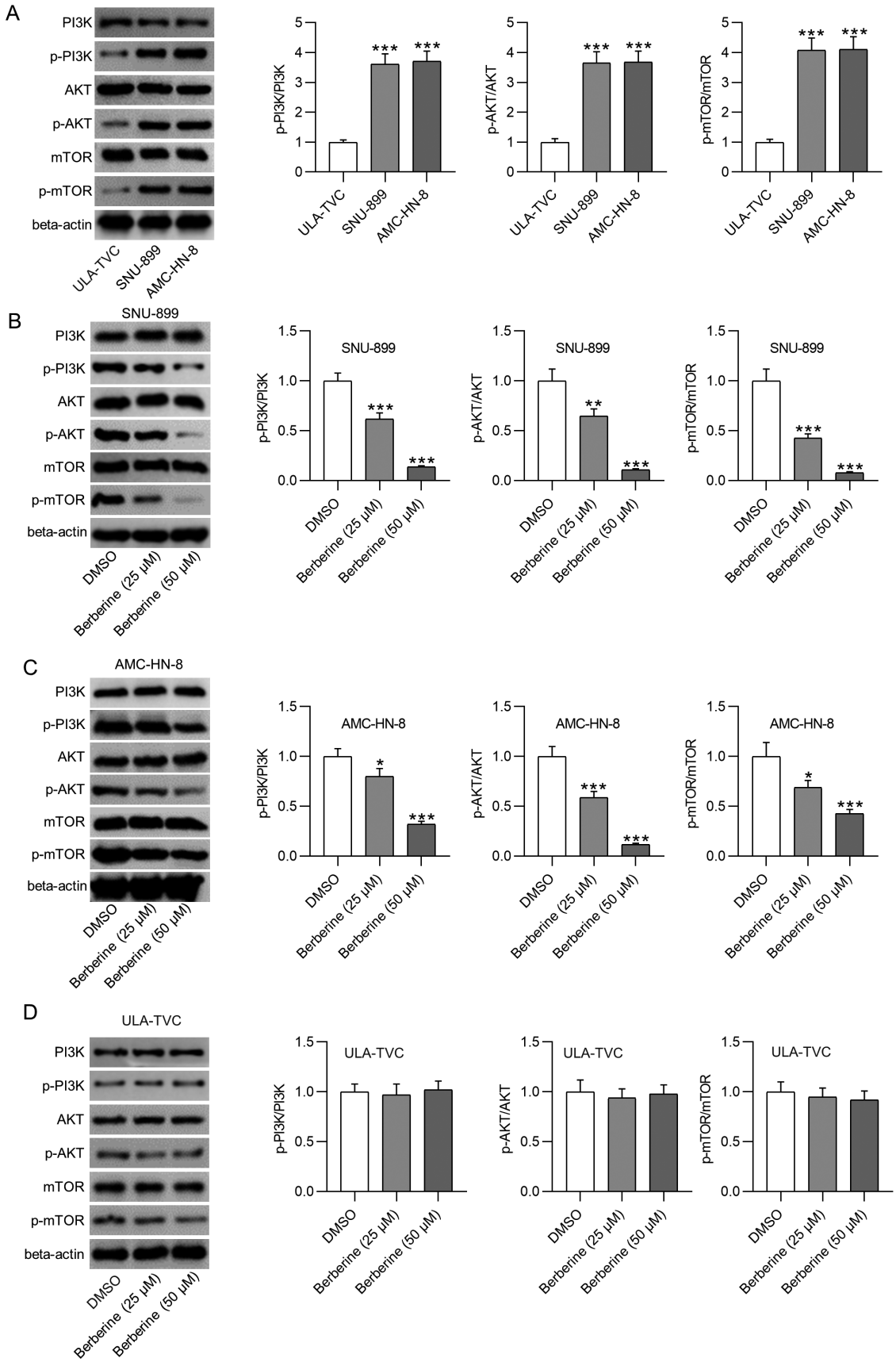

Fig. 4. Berberine inactivates PI3K/AKT/mTOR signaling in LSCC cells. **A.** Measurement of the protein levels of PI3K, AKT, mTOR, p-PI3K, p-AKT, and p-mTOR in ULA-TVC, AMC-HN-8, and SNU-899 cells. **B, C.** Phosphorylated and unphosphorylated PI3K, AKT, and mTOR proteins were analyzed by western blot in AMC-HN-8 and SNU-899 cells treated with berberine (25 or 50 μM). **D.** Detection of the effect of berberine on PI3K, AKT, mTOR, p-PI3K, p-AKT, and p-mTOR protein levels in ULA-TVC cells. **P*<0.05, ***P*<0.01, ****P*<0.001.

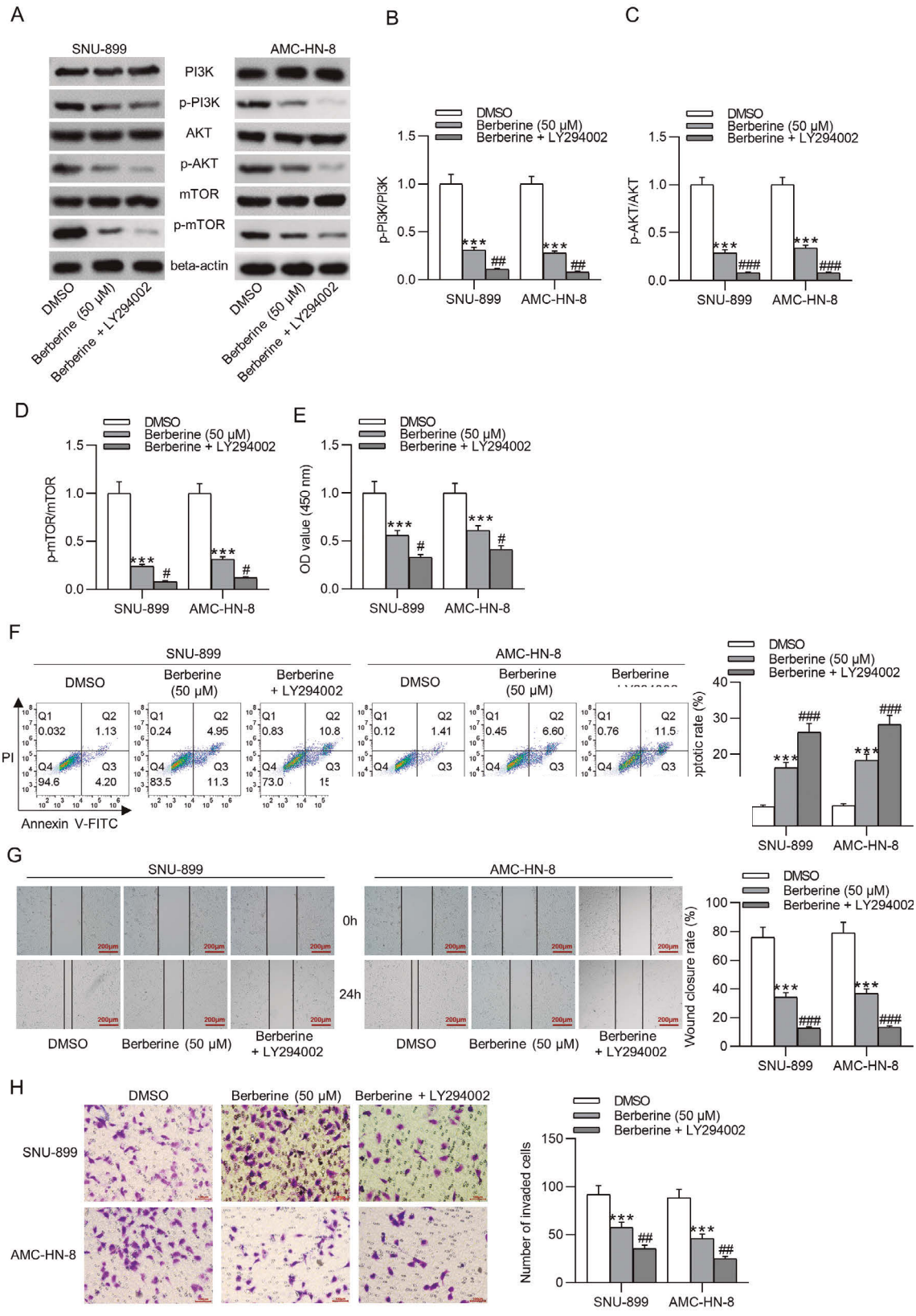

Fig. 5. LY294002 enhances the inhibitory effects of berberine on malignant cell behavior. **A-D.** Protein levels of PI3K, AKT, mTOR, p-PI3K, p-AKT, and p-mTOR in AMC-HN-8 and SNU-899 cells treated with berberine (50 μM) and LY294002 (50 μM) were detected by western blot. **E.** Cell viability of AMC-HN-8 and SNU-899 cells treated with berberine (50 μM) and LY294002 (50 μM) were determined by the CCK-8 assay. **F.** Cell apoptosis of AMC-HN-8 and SNU-899 cells treated with berberine (50 μM) and LY294002 (50 μM) were measured by flow cytometry. **G, H.** Cell migration and invasion of AMC-HN-8 and SNU-899 cells treated with berberine (50 μM) and LY294002 (50 μM) were tested by the wound healing and Transwell assays, respectively. ***P<0.001 vs. DMSOgroup. $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. berberine (50 μM) group.

PI3K/AKT/mTOR signaling in LSCC cells.

LY294002 enhances the inhibitory effects of berberine on malignant cell behavior

To validate the effect of berberine on PI3K/AKT/mTOR signaling, LY294002 (an inhibitor of PI3K) was used to treat LSCC cells. As shown in Fig. 5A-D, the inhibitive effect of berberine on the phosphorylation of the PI3K, AKT, and mTOR proteins was strengthened by LY294002 treatment. Moreover, the berberine-mediated decrease in cell viability was further reduced by LY294002 treatment (Fig. 5E). Additionally, the berberine-mediated enhancement of the cell apoptosis rate was also augmented by LY294002 treatment (Fig. 5F). The suppressive effect of berberine on cell migration and invasion is enhanced by LY294002 treatment (Fig. 5G,H). In summary, LY294002 enhances the inhibitory effects of berberine on malignant cell behavior.

The regulatory role of berberine in LSCC cells

Berberine exerts inhibitive effects on the phosphorylation of PI3K, AKT, and mTOR, and then facilitates cell apoptosis but attenuates cell growth, migration, and invasion. Notably, LY294002 enhanced these inhibitive effects.

Discussion

With high recurrence and distant metastasis, LSCC is difficult to cure, leading to an unsatisfied life and a poor prognosis for LSCC patients after surgery (Ouban, 2022; Zhao et al., 2022). Therefore, exploring effective medicines is of great significance for LSCC treatment. In the present study, the biological functions and potential mechanisms of berberine were investigated in LSCC for the first time. Specifically, berberine facilitated cell apoptosis but attenuated cell proliferation, migration, and invasion. Moreover, berberine significantly suppressed the activation of PI3K/AKT/mTOR signaling by inhibiting the phosphorylation of PI3K, AKT, and mTOR. Importantly, LY294002 treatment enhanced the effects of berberine on the cell phenotype and PI3K/AKT/mTOR signaling.

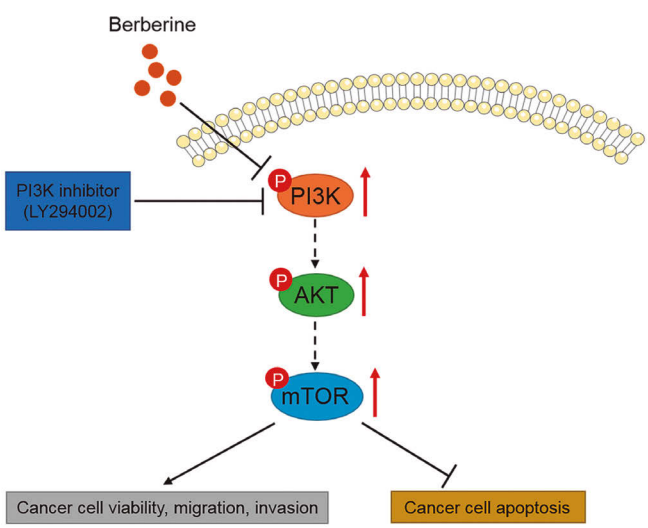

Fig. 6. The regulatory role of berberine in LSCC cells. Berberine exerts an inhibitive effect on the phosphorylation of PI3K, AKT, and mTOR, and then facilitates cell apoptosis but attenuates cell growth, migration, and invasion in LSCC. Notably, LY294002 enhanced these inhibitive effects.

Excessive tumor cell growth and reduced cell apoptosis are typical characteristics of tumor progression (Kroemer and Pouyssegur, 2008; Hayes et al., 2020). Notably, berberine has been widely reported to modulate cell growth and apoptosis in various cancers, including glioblastoma, gastric cancer, and bladder cancer (Eom et al., 2010; Yan et al., 2011; Peng et al., 2022). In detail, berberine suppressed cell growth by inactivating the PP2A/MCL-1 pathway (Peng et al., 2022). Also, berberine induced G1 cell cycle arrest and accelerated cell apoptosis in bladder cancer (Yan et al., 2011). Furthermore, berberine enhanced apoptosis of glioblastoma T98G cells by targeting the mitochondrial/caspase pathway (Eom et al., 2010). In our exploration, berberine also inhibited cell growth and promoted apoptosis in LSCC cells.

Tumor cell migration and invasion are critical steps of tumor metastasis (Kerndt et al., 2021; Bayır et al., 2022). Importantly, berberine could inhibit cell migration and invasion during cancer progression (Li et al., 2021, 2023). According to a previous study, berberine attenuated cell migration and invasion by inhibiting Notch1/PTEN/PI3K/AKT/mTOR in colon cancer cells (Li et al., 2021). Moreover, Berberine exerted an inhibitive effect on the migration and invasion of bladder cancer cells via inactivating the HER2/PI3K/AKT pathway (Li et al., 2023). Similarly, our research also revealed that berberine hampered cell migration and invasion in LSCC.

Mechanistically, berberine was identified to target PI3K/AKT/mTOR signaling pathways in multiple tumor cells (Kou et al., 2020; Shi et al., 2023). For instance, berberine enhanced cell apoptosis in gastric cancer by repressing the PI3K/AKT/mTOR pathway (Kou et al., 2020). Besides, berberine exerts anti-tumor activity in CAL-62 and BHT-101 cells by activating autophagy and apoptosis via PI3K/AKT/mTOR signaling (Shi et al., 2023). In our study, berberine also inactivated PI3K/AKT/mTOR signaling by downregulating phosphorylated PI3K, AKT, and mTOR protein levels in LSCC cells. Moreover, treatment with the PI3K inhibitor (LY294002) strengthened the effect of berberine on cell phenotype and PI3K/AKT/mTOR signaling.

In summary, we further revealed the role of berberine in LSCC. The experimental results showed that berberine inhibits the LSCC malignant cell phenotype by inactivating PI3K/AKT/mTOR signaling. Our study may provide a novel strategy for LSCC

management. However, this study had limitations. First, the role of berberine *in vivo* was not studied. Second, the effect of berberine on other cellular processes and signaling pathways was missing. We believe that these issues will be addressed in the future.

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Conflicts of interest. The authors declare that there are no competing interests in this study.

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