Article

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Baicalein Inhibits Metastasis of Oral Squamous Cell Carcinoma Cells by Regulating ERK/ELK-1/Snail Signaling

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Background: Oral squamous cell carcinoma (OSCC) is associated with high recurrence and poor prognosis. Baicalin has multiple pharmacological effects, including anti-inflammatory and anti-proliferative activities. Here, we examine the effect of baicalein on OSCC metastasis and its potential mechanism of action.
Methods: SCC-4 and CAL-27 cells were treated with different concentrations of baicalein. The proliferation of OSCC cells was evaluated by Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. As for migration and metastasis, baicalein-treated OSCC cells were used for wound healing assay and Transwell assay. The levels of epithelial-mesenchymal transition-related proteins (E-cadherin, N-cadherin, vimentin) and extracellular regulated protein kinases (ERK)/ETS Transcription Factor ELK1 (ELK-1)/Snail signaling pathway-related proteins in baicalein-treated OSCC cells were evaluated by western blotting.
Results: The rates of cell proliferation and migration, along with the metastatic potential, of baicalein-treated cells were significantly lower than those of the control ($p < 0.05$), and the effects were concentration-dependent. Furthermore, compared to the control, baicalein significantly decreased the levels of N-cadherin and vimentin in SCC-4 and CAL-27 cells, and increased the E-cadherin level ($p < 0.05$). Mechanistically, baicalein downregulated the levels of p-ERK1/2, phospho-ETS Transcription Factor ELK1 (p-ELK-1), and Snail ($p < 0.05$). Finally, the ERK/ELK-1/Snail pathway inhibitor (U0126) promoted the effect of baicalein in inhibiting the migration and invasion of OSCC cells ($p < 0.05$).
Conclusion: Baicalein abates the migration, invasion, and metastasis of OSCC cells through the ERK/ELK-1/Snail signaling pathway. This study provides a basis for the development of baicalein as a compound for the treatment of OSCC.

Keywords: baicalein; oral squamous cell carcinoma; cell metastasis; ERK/ELK-1/Snail

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Introduction

As one of the most common malignant tumors, the pathogenesis of oral squamous cell carcinoma (OSCC) is very complex, which may be the result of many factors such as poor eating habits, smoking, drinking, environmental effects and virus infections [1,2]. At present, the most effective treatment for patients with oral cancer is surgery, whereas patients have no obvious symptoms in the early stage of the disease. When most patients are treated, the disease has progressed to the middle and late stages. The effect of surgical resection on patients is not ideal. Postoperative chemotherapy is usually required, but due to individual differences in patients, some patients do not respond to chemotherapy drugs [3]. The five-year survival rate of patients with oral cancer ranges from 50% to 55% in the past 20 years, with no significant improvement, which is due to the occurrence of distant metastasis and tumor invasion [4]. Furthermore, in-depth analysis of the mechanisms of metastasis and invasion in this population and the search for new chemotherapeutic drugs are the key problems to be solved.

Baicalein, a natural flavonoid, has been isolated from the root of *Scutellaria baicalensis* Georgi. Baicalein has strong inhibitory outcomes on nasopharyngeal carcinoma [5], non-small cell lung cancer [6], glioma [7], thyroid cancer [8], and other malignancies. Its mechanism of action mainly includes inhibition of tumor cell growth, induction of apoptosis and autophagy, and enhancement of tumor cell sensitivity to chemotherapeutic drugs [9]. Gao *et al.* [10] reported that baicalein can effectively abate OSCC cell growth and induce apoptosis. In other studies, tumor-derived C-X-C motif chemokine ligand 5 (CXCL5), through activation of extracellular regulated protein kinases (ERK)/ETS Transcription Factor ELK1 (ELK-1)/Snail signaling, can promote colorectal cancer metastasis [11], which is supported by the finding that baicalein can abate colon cancer cell metastasis through the ERK signaling pathway [12]. However, the mechanism of action of baicalein in regulating ERK signaling in OSCC remains un-

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clear. In view of these findings, SCC-4 and CAL-27 cells (human OSCC cells) are used in this study, which aims to evaluate the effects of baicalein on OSCC cell migration and invasion and to define the potential molecular mechanism, so as to provide a strong basis for the use of baicalein as a lead compound for drug development.

Materials and Methods

Cell Culture

Human OSCC SCC-4 (catalog number: CC-Y1750) and CAL-27 (catalog number: NM-KL01) cell lines were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). An approved DNA-based method was used to confirm the origins of both cell lines, and each cell line name was checked against the ICLAC database for mistakenly identified cells. Short tandem repeat (STR) information was applied to obtain the genetic characteristics, and mycoplasma testing was performed to confirm the absence of contamination. Cells were cultured in DMEM (11995065, Thermo Scientific, Waltham, MA, USA), containing 10% fetal bovine serum (FBS, C0235, Thermo Scientific, Waltham, MA, USA), 100 μg·mL^{-1} streptomycin (15140122, Thermo Scientific, Waltham, MA, USA), and 100 μg·mL^{-1} penicillin (A9314, Thermo Scientific, Waltham, MA, USA), in 5% CO$_2$ at 37 °C and passaged every 2–3 days. Cells in the logarithmic growth phase were used for all experiments. ERK1/2 inhibitor U0126 (Cell Signaling Technology, 109511-58-2, Danvers, MA, USA, U0126 was dissolved in DMSO and stored at –20 °C, and then diluted to the final concentration (10 μmol/L) with fresh culture medium as needed before the experiment).

Methylthiazolyldiphenyl-Tetrazolium Bromide (MTT) Assay

Cells in the logarithmic growth phase were adjusted to 6 × 10^4 cells/mL and seeded into a 96-well plate, with 50 μL of the cell suspension added to each well. Different concentrations (0, 5, 10, 20, 40, 80, 100 mmol·L^{-1}) of baicalein was added to each well after 24 h of incubation. Cells were incubated for 48 h, and 20 μL of MTT solution (M1025, Sigma, St. Louis, MO, USA) was added to each well at a concentration of 5 mg·mL^{-1}. The supernatant was discarded after incubation for 4 h and 100 μL of DMSO (67-68-5, Solarbio, Beijing, China) was added to each well, followed by shaking for 10 min. Finally, an enzyme labeler (A51119600C, Thermo Fisher, Waltham, MA, USA) was added to each well, and the absorbance values were measured at 570 nm.

Wound Healing Assay

Cells in the logarithmic growth phase were adjusted to 5 × 10^5 cells/mL and seeded in a 6-well plate, with 2 mL of the cell suspension added to each well. The plate was incubated for 24 h to allow the formation of a confluent monolayer. A sterile 200-μL pipette tip was used to create a straight line down the center of each well. Cells were rinsed with sterile PBS (AM9624, Sigma, Milwaukee, WI, USA), and different concentrations (40, 80, 100 μmol·L^{-1}) of baicalein were added. At 0, 24, and 48 h, cells were examined with an inverted microscope (ECLIPSE Ts2, Nikon, Tokyo, Japan), and the cell migration rate was calculated.

Transwell Assay

OSCC cells treated with different concentrations (40, 80, 100 μmol·L^{-1}) of baicalein were collected, resuspended in serum-free DMEM (11965126, Wuhan Punosai Life Technology Co., Ltd., Wuhan, China), and counted. For the Transwell assay, the upper compartment of each chamber received 200 μL of the cell suspension containing 5 × 10^4 cells, while the lower chamber received 600 μL of medium containing 10% FBS. The media in the upper and lower chambers was discarded after 24 h of incubation, and the membrane was removed and stained with 0.1% crystal violet solution (G1064, Solarbio, Shanghai, China) for 10 min after fixation with methanol for 20 min. Finally, cells were removed with a cotton swab. After the cells were air-dried, they were photographed with an inverted microscope (ECLIPSE Ts2, Nikon, Tokyo, Japan), and the number of cells was counted in six randomly selected fields. For the invasion assay, the membrane was precoated with Matrigel before adding 200 μL of the cell suspension containing 1 × 10^5 cells.

Western Blotting

Cells in the logarithmic phase were adjusted to 1 × 10^5 cells/mL and added to 10-cm cell culture dishes, with 10 mL of the cell suspension per dish. After 24 h of incubation, baicalein (40, 80, 100 μmol·L^{-1}) was added. Cells were collected after 48 h of incubation and resuspended in RIPA buffer (P0013B, Millipore, Billerica, MA, USA), containing protease inhibitors and phosphatase inhibitors. Cells were sonicated on ice for 30 min and centrifuged at 12,000 r·min^{-1} for 10 min. Supernatants were collected and protein concentration was determined by the BCA method (P0012, Beyotime, Shanghai, China). Next, proteins (30 μg) were combined with 5× sample buffer, denatured at 95 °C for 10 min, and separated by 10% SDS-PAGE. Proteins were electrotransferred to nitrocellulose membranes, which were subsequently blocked at room temperature for 1 h with 5% skimmed milk. The following primary antibodies were used: E-cadherin (1:1000, ab233611, Abcam, Shanghai, China), N-cadherin (1:1000, ab254512, Abcam, Shanghai, China), vimentin (1:1000, ab92547, Abcam, Shanghai, China), phospho-extracellular regulated protein kinases 1/2 (p-ERK1/2, 1:1000, ab126445, Abcam, Shanghai, China), ERK1/2 (1:1000, ab176640, Abcam, Shanghai, China), phospho-ETS Transcription Factor ELK1 (p-ELK-1, 1:1000, ab218133, Abcam, Shanghai, China), ELK-1

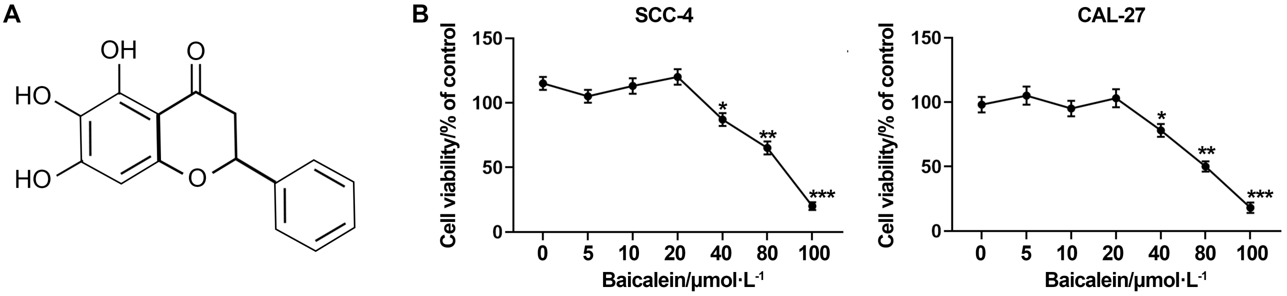

Fig. 1. Effect of baicalein on oral squamous cell carcinoma (OSCC) cell proliferation. (A) Chemical structure of baicalein. (B) Effect of baicalein on cell activity (SCC-4 and CAL-27) through Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control, n = 3.

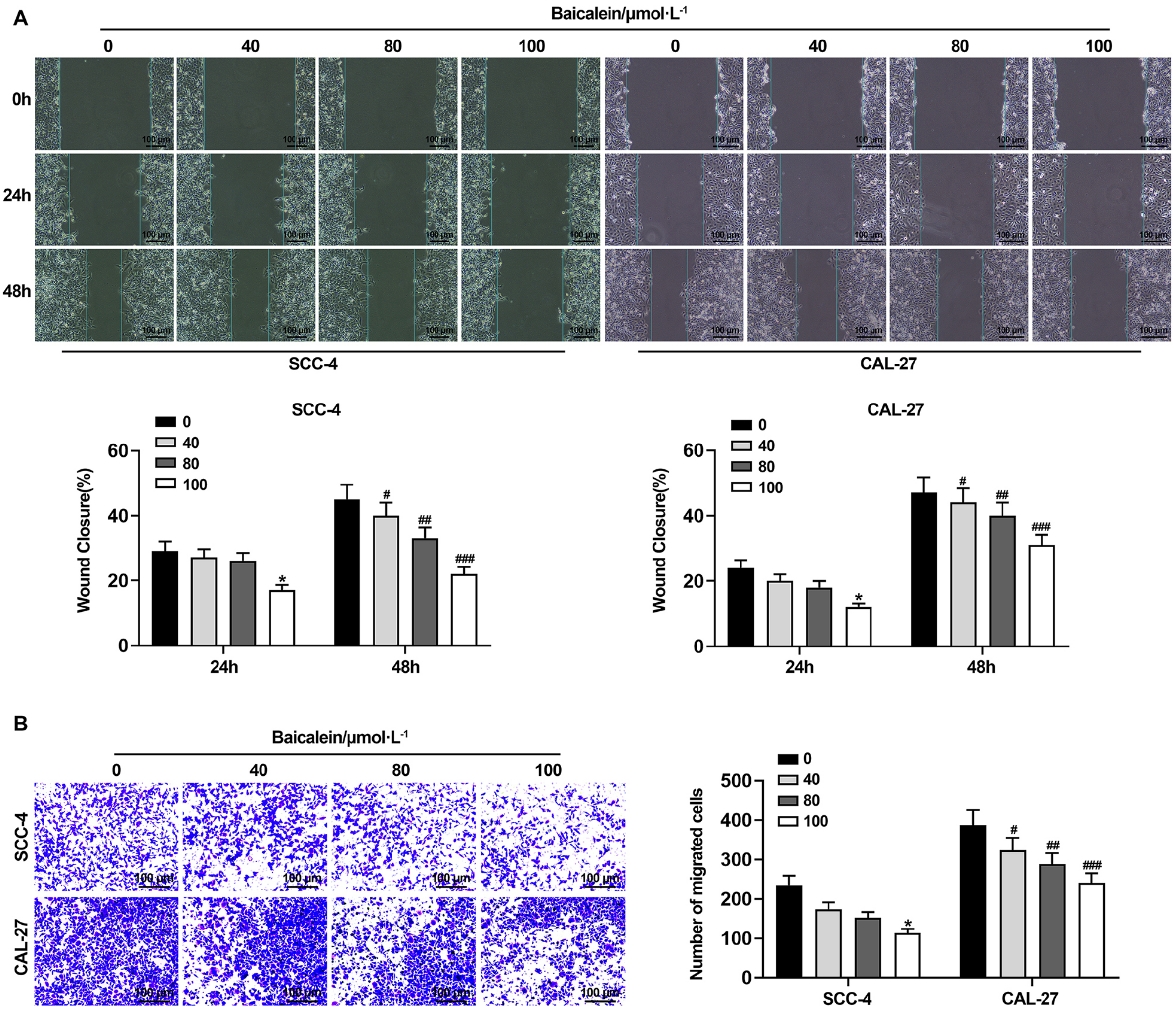

Fig. 2. Effect of baicalein on the migration of SCC-4 and CAL-27 cells. (A) Anti-migratory effect of baicalein was evaluated through wound healing assay. *$p < 0.05$ versus control, at 24 h; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ versus control, at 48 h. Scale bar: 100 μm. (B) Anti-migratory effect of baicalein was evaluated through Transwell assay. Scale: 100 μm. *$p < 0.05$, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ versus control, n = 3.

(1:1000, ab188316, Abcam, Shanghai, China), and Snail (1:1000, ab216347, Abcam, Shanghai, China) overnight at 4 °C. Membranes were then incubated in goat anti-rabbit IgG (Wuhan Sanying Biotechnology Co., Ltd., A11034, Wuhan, China) for 2 h, washed three times with PBST for 10 min each time. The target proteins were visualized using the ECL chemiluminescence kit (P0018S, Beyotime, Shanghai, China) and immunoblot imaging system

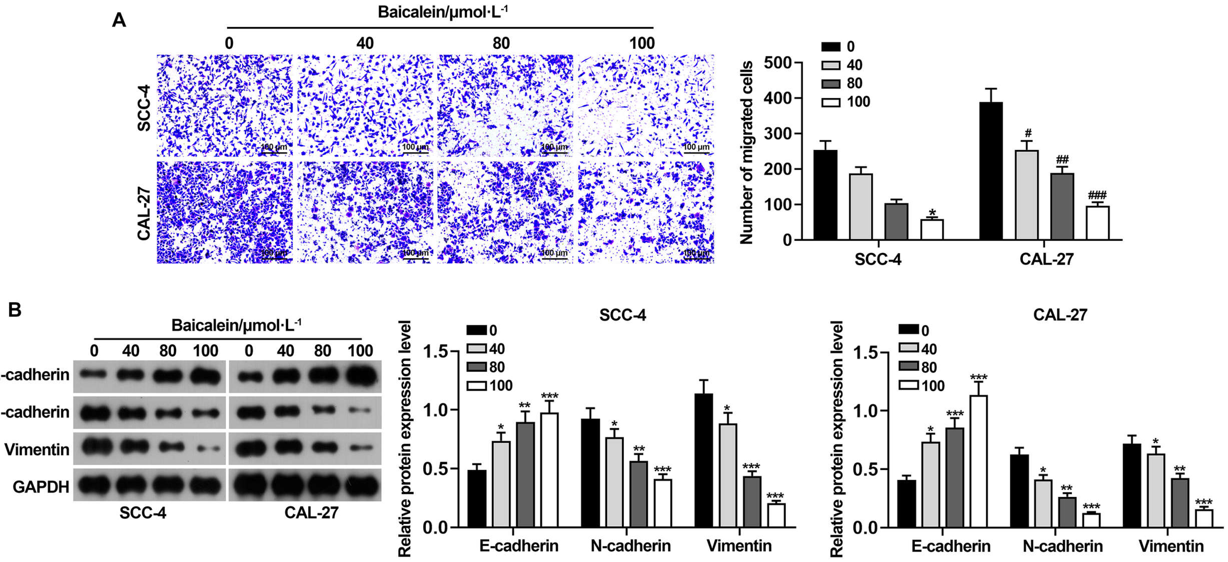

Fig. 3. Effect of baicalein on the invasion of OSCC cells. (A) SCC-4 and CAL-27 cells were treated with baicalein (0, 40, 80, 100 μmol·L^{-1}) for 48 h, and cell invasion was evaluated through Transwell assay. *$p < 0.05$, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ versus control. Scale bar: 100 μm. (B) Levels of epithelial-mesenchymal transition-related proteins (E-cadherin, N-cadherin, vimentin) were measured through western blotting. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control, n = 3. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(iBright FL1000, Thermo Fisher, Waltham, MA, USA). ImageJ software (ImageJ2, NIH, Bethesda, MA, USA) was used to analyze the target proteins.

Statistical Analyses

GraphPad Prism 8.0 software was used for statistical analyses (GraphPad Software, San Diego, CA, USA). Each experiment was repeated three times, and all data were expressed as $\bar{x} \pm$ s. Comparisons between the two groups were made using the independent samples *t*-test. One-way analysis of variance was used for multiple comparisons. Tukey test was used for post hoc testing. $p < 0.05$ was considered as a statistically significant difference.

Results

Effect of Baicalein on the Proliferation of OSCC Cells

To investigate the impact of baicalein on the viability of OSCC cells, SCC-4 and CAL-27 cell lines were exposed to varying concentrations of baicalein (0–100 μmol·L^{-1}). The viability of these cells was then evaluated after 48 hours using the MTT assay (Fig. 1A). It was observed that baicalein concentrations up to 40 μmol·L^{-1} did not significantly affect the proliferation rates of SCC-4 and CAL-27 cells (Fig. 1B, $p < 0.05$). Conversely, higher concentrations of baicalein, ranging from 40 to 100 μmol·L^{-1}, resulted in a notable suppression of OSCC cell growth in a dose-dependent manner (Fig. 1B, $p < 0.05$)..

Effect of Baicalein on OSCC Cell Migration

At concentrations of 40, 80, and 100 μmol·L^{-1}, baicalein significantly inhibited cell migration (SCC-4 and CAL-27) compared with control, which was validated through wound healing assay (Fig. 2A, $p < 0.05$). In the Transwell assay, different concentrations of baicalein (40, 80, and 100 μmol·L^{-1}) significantly reduced the number of cells that migrated through the membrane compared with control (Fig. 2B, $p < 0.05$). These results indicate that baicalein can inhibit the migration of SCC-4 and CAL-27 cells.

Effect of Baicalein on OSCC Cell Invasion

OSCC cells were plated into Transwell chambers precoated with Matrigel and cultured for 24 h, followed by treatment with baicalein (40, 80, 100 μmol·L^{-1}). The number of cells that migrated through the membrane of the Transwell assay was significantly and dose-dependently lower in the baicalein-treated subgroup than that in the control, indicating that baicalein reduced the invasive ability of OSCC cells (Fig. 3A, $p < 0.05$). Subsequently, the levels of epithelial mesenchymal transition-related proteins (E-cadherin, N-cadherin, vimentin) were evaluated through western blotting, and the E-cadherin level was increased in the baicalein-treated subgroup, while the levels of N-cadherin and vimentin were decreased compared with control (Fig. 3B, $p < 0.05$).

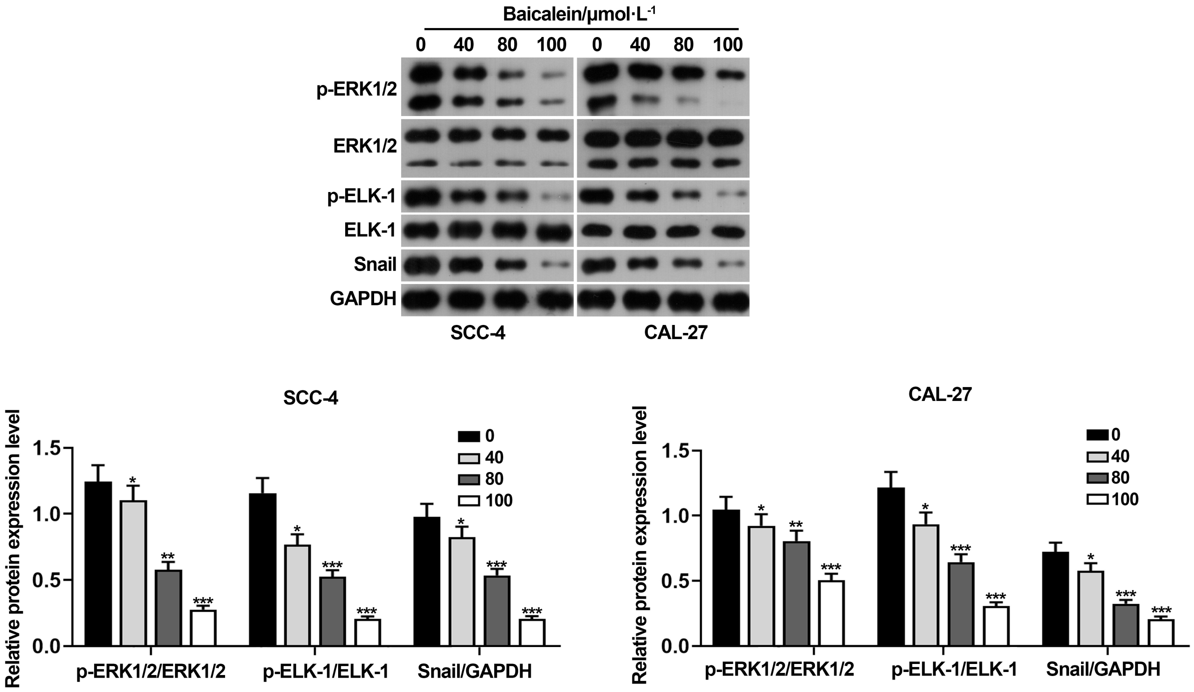

Fig. 4. Baicalein abates extracellular regulated protein kinases (ERK)/ETS Transcription Factor ELK1 (ELK-1)/Snail signaling in OSCC. Western blotting was used to evaluate the levels of ERK/ELK-1/Snail signaling pathway proteins phospho-extracellular regulated protein kinases 1/2 (p-ERK1/2), ERK1/2, phospho-ETS Transcription Factor ELK1 (p-ELK-1), ELK-1, and Snail. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control, n = 3.

Baicalein Abates ERK/ELK-1/Snail Signalling in OSCC

ERK1/2 signaling pathway, which is abnormally activated in cancer, facilitates OSCC cell migration, invasion, and metastasis. Western blotting was used to examine the effect of baicalein on the total and phosphorylated levels of proteins within the ERK1/2 signaling pathway. Baicalein abated the phosphorylated ERK1/2 level in a concentration-dependent manner, along with phosphorylated ELK-1 and Snail levels (Fig. 4, $p < 0.05$).

Baicalein Alleviates OSCC Cell Migration, Invasion, and EMT Progression by Abating ERK/ELK-1/Snail Signaling

SCC-4 cells were treated with the ERK1/2 inhibitor U0126. In the baicalein+U0126 subgroup, the phosphorylated levels of ERK1/2, ELK-1, and Snail were significantly abated compared to the control. N-cadherin and vimentin protein levels were decreased, while the E-cadherin protein level was increased in the baicalein+U0126 subgroup compared with control (Fig. 5A, $p < 0.05$). These results were validated through Transwell assay, which showed that SCC-4 cell migration and invasion were significantly reduced in the baicalein+U0126 subgroup compared to the control (Fig. 5B, $p < 0.01$). This indicates that baicalein alleviates OSCC cell migration, invasion, and Epithelial-Mesenchymal Transition (EMT) progression by abating ERK/ELK-1/Snail signaling.

Discussion

Uncontrolled regulation of cell proliferation and cell survival is one of the main factors in the development and progression of cancer [13]. Because tumor cells can metastasize and invade local and distant tissues, it is difficult to completely eradicate tumors. Preoperative induction chemotherapy for cancer patients, which causes less local vascular damage and requires good blood supply, is conducive to the effectiveness of most chemotherapy drugs [14]. OSCC is a challenging disease to treat and a serious worldwide problem. Due to the limitations of its treatment, the survival rate of patients with OSCC remains unacceptable. The neoadjuvant chemotherapy regimen, which involves using DDP in combination with 5-Fu, is the first choice for preoperative induction chemotherapy of OSCC [15]. However, it is difficult to measure certain physiological indexes in clinical practice, which makes it difficult to objectively evaluate its efficacy and the side effects of chemotherapy drugs that often greatly reduce the quality of life of patients [16]. Therefore, it is important to identify new therapeutic drugs and new therapeutic targets. This study found that baicalein can significantly abate the migration and invasion of OSCC cells, suggesting that the active ingredient of this traditional Chinese medicine may become a drug for the treatment of OSCC in the future.

In recent years, identifying effective components from natural medicines has gained enormous research momen-

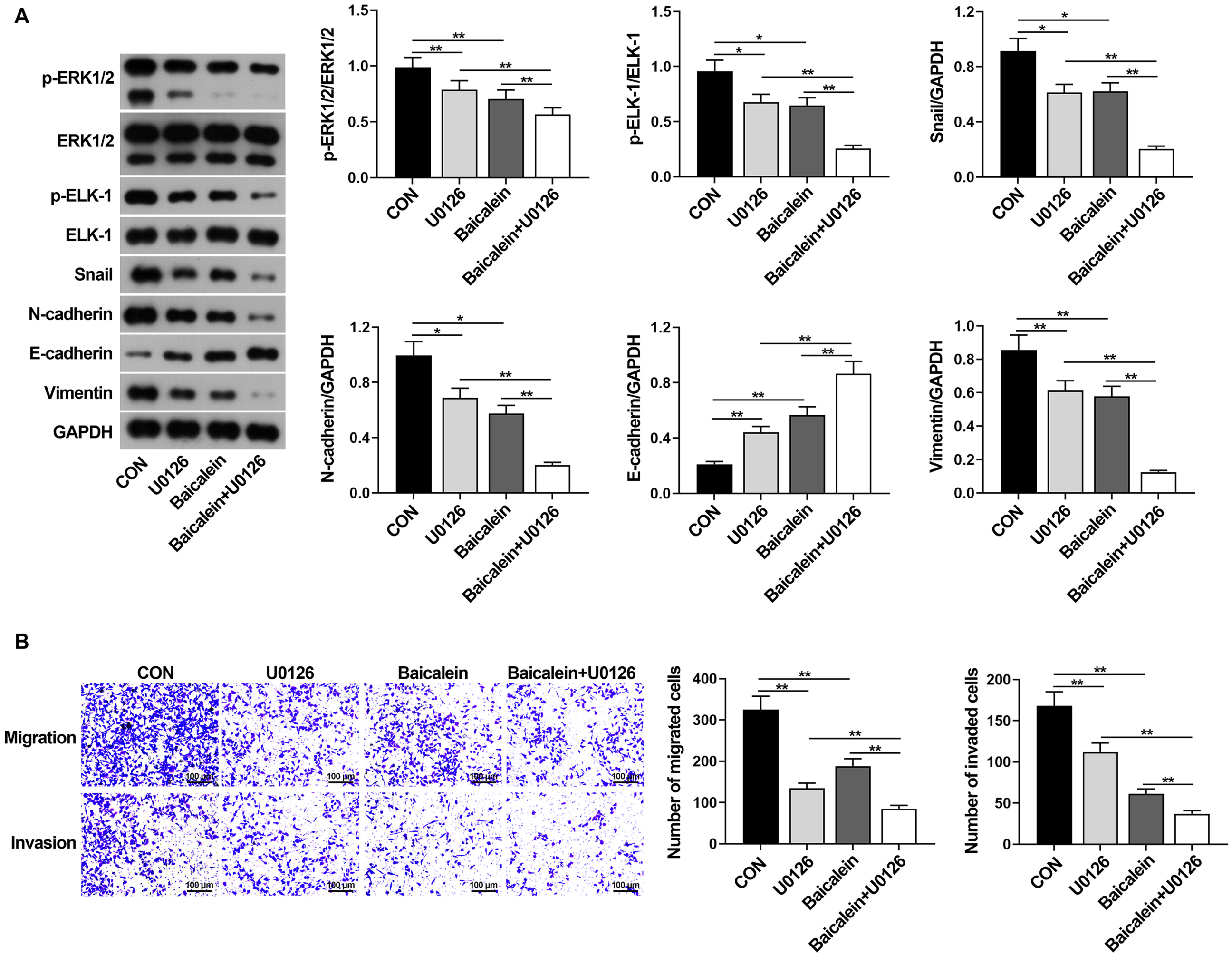

Fig. 5. Baicalein alleviates OSCC cell migration, invasion, and Epithelial-Mesenchymal Transition (EMT) progression by abating ERK/ELK-1/Snail signaling. SCC-4 cells were treated with the ERK1/2 inhibitor U0126 in combination with baicalein (100 mmol/L^{-1}). (A) Western blotting was used to evaluate the levels of ERK/ELK-1/Snail pathway proteins (p-ERK1/2, ERK1/2, p-ELK-1, ELK-1, Snail) and epithelial-mesenchymal transition-related proteins (E-cadherin, N-cadherin, vimentin). (B) Transwell assay was used to evaluate the migration and invasion of SCC-4 cells. Scale bar: 100 μm. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control, n = 3.

tum. Baicalein is mainly present in *Scutellaria baicalensis* Georgi and is the main active ingredient in the root of *Scutellaria baicalensis* Georgi [17]. Baicalein has a wide range of pharmacological effects. It improves cerebral blood circulation, reduces cerebrovascular resistance and anti-platelet aggregation, and protects neurons, along with exert anti-inflammatory, anti-HIV, anti-oxidative, antibacterial, anti-allergic, and anti-diabetic activities [18,19]. A previous study has found that baicalein also has a wide range of therapeutic effects on lung cancer, breast cancer, gastric cancer, colon cancer, prostate cancer, and other malignancies [20]. Gao *et al.* [10] reported that baicalein can effectively abate the growth of OSCC cells and induce apoptosis through the Sp1/nuclear factor kappa-B (NF-κB) signaling pathway. In this study, we found that different concentrations of baicalein abated the activity of OSCC cells, thereby decreasing cell migration, invasion, and EMT. In addition, baicalein can effectively abate the migration, invasion, and metastasis of various malignancies such as liver cancer [21], breast cancer [22], and prostate cancer [23]. These studies show that baicalein has a good therapeutic effect in various cancers.

ERK signaling is abnormally activated in various cancers, including OSCC. Abnormally activated ERK promotes the growth, invasion, and metastasis of tumor cells by activating downstream targets such as ELK-1 [24]. Li *et al.* [25] confirmed that the expression of p-ERK1/2 is upregulated in OSCC cells, and the use of the small molecule inhibitor U0126, which specifically blocks the MAPK/ERK signaling pathway, can significantly abate the migration and invasion of OSCC cells. In this study, we found that baicalein did not change the expression of total ERK1/2 protein, but significantly reduced the levels of phosphorylated ERK1/2 and ELK-1. In addition, the use of

U0126 not only significantly enhanced the inhibitory effect of baicalein on p-ERK1/2 (T202/Y204), p-ELK-1 (S383), Snail, and mesenchymal cell markers N-cadherin and vimentin, but also augmented the upregulation of the epithelial cell marker protein E-cadherin by baicalein. This indicates that baicalin inhibits EMT in OSCC cells through the ERK/ELK-1/Snail signaling pathway, thereby abating the migration, invasion, and metastasis of OSCC cells. However, this study has certain limitations. U0126 is an ERK pathway antagonist, and we have not yet identified a specific ERK/ELK-1/Snail pathway antagonist. Further research is needed to investigate other pathways in the future.

Conclusion

In conclusion, this study found that baicalein can effectively abate the migration, invasion, and metastasis of OSCC cells, and the mechanism of action may involve the inhibition of EMT in OSCC cells through the ERK/ELK-1/Snail signaling pathway. These findings suggest that baicalein possesses potent anti-metastatic properties against OSCC, thereby providing a substantial foundation for its potential application in clinical treatments for OSCC.

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

Author Contributions

FL drafted the manuscript and revised it for important intellectual content. YSH and FL collected data and prepared it for the manuscript. YSH analyzed the data, participated in constructive discussions, and supervised the project. Both authors contributed significantly to editorial changes of important content. Both authors read and approved the final manuscript. Both authors have agreed to be accountable for all aspects of the work and to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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