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Cryptotanshinone inhibits oral squamous cell carcinoma through the autophagic pathway

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Oral squamous cell carcinoma (OSCC) is one of the most common malignant tumors with a low quality of life. Because traditional surgical treatment often causes large wounds and then affects the quality of life of patients, it is urgent to find new and efficient drugs with good safety for clinical treatment. This study aimed to identify potential anticancer drugs starting from the traditional Chinese medicine *Salvia miltiorrhiza* extract. Cryptotanshinone, a compound isolated from the Chinese herb *Salvia miltiorrhiza*, was found to significantly induce apoptosis and inhibit proliferation in OSCC. By electron microscopy, autophagosomes were found. Confocal fluorescence microscopy data showed that cryptotanshinone significantly induced autophagy in OSCC cells. Mechanistically, the western blot assay indicated that cryptotanshinone induced cell autophagy through the activation of the LC3 pathway, whereas the autophagy inhibitor 3-methyladenine attenuated these effects. Furthermore, we demonstrated that cryptotanshinone had a significant antitumor effect in a tumor xenograft model, and no damage to vital organs was observed. Our findings provide evidence that cryptotanshinone may be a novel therapeutic strategy for the treatment of OSCC.

Key words: autophagy; cryptotanshinone; apoptosis; oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC), originating from sites such as the alveolar ridge, buccal mucosa, floor of the mouth, palate, and tongue, is the sixth most common malignancy worldwide, mainly tongue squamous cell carcinoma (TSCC), accounting for the vast majority of ~377,713 new cases and ~177,757 deaths in 2020 in the world. Most global OSCC cases are diagnosed in Asia [1]. A high rate of lymph node metastasis was found in the early stage of the tumor [2]. Its overall 5-year survival rate is less than 60% due to high invasiveness and resistance to treatments such as radiotherapy, chemotherapy, or combination therapy [3, 4]. The failure of chemotherapy and radiotherapy leads to tumor recurrence and poor prognosis, mainly due to limited efficiency and side effects, for example, conventional chemotherapy targets both cancer cells and normal cells, resulting in toxic effects on the body [5, 6]. At present, tumor resection is still the main strategy for oral cancer treatment [7], thus the search for more stable, effective, and safe anticancer drugs has become an urgent priority.

Natural products are an important resource for new drug candidates, especially anticancer drugs [8, 9], and about 80–83% of approved anticancer drugs are natural drugs or mimic natural products [10]. As bioactive components of natural products, vincristine, paclitaxel, and curcumin have historically been used in clinical cancer therapy [8, 11]. By screening novel chemotherapeutic drugs from natural products, we identified cryptotanshinone as a potent anticancer candidate from a natural product-based library consisting of diverse compounds [12]. Cryptotanshinone, an active component of the Chinese herbal medicine *Salvia miltiorrhiza*, which is derived from the root and rhizome of *Salvia miltiorrhiza*, is a major lipid-soluble extract of *Salvia miltiorrhiza*, which is a diterpenoid quinone [12].

Danshen extract has many biological effects, including anticancer, anti-inflammatory, immunomodulatory, neuroprotective, and antifibrotic activities, partly by regulating JAK2/STAT3 and TLR4-MyD88/PI3K/Nrf2 and PI3K/Akt eNOS pathways [13–15].

However, until now, the biological function of cryptotanshinone in oral cancer treatment has rarely been reported, and its molecular mechanism remains largely unknown.

Autophagy is a steady-state and catabolic degradation reaction of cells. It begins with the formation of double-membrane vesicles and phagocytosis of proteins, cytoplasm, protein aggregates and organelles, and then transports them to lysosomes, where they are degraded for use as alternative energy sources [16]. Autophagy plays a dual role in tumorigenesis, and although some studies have reported that autophagy plays a pro-survival role, most drug-induced autophagy mainly leads to cell death in cancer [17–20]. Autophagy can reduce the survival time of tumor cells, leading to apoptosis [16, 21]. Recent evidence suggests that promoting autophagy can increase the chemosensitivity of tumor cells to drugs, leading to tumor cell death [22]. Therefore, the promotion of autophagy by anticancer drugs has been recognized as an important component of cancer therapy [16, 21]. We have examined cryptotanshinone in the context of cancer therapy and have shown that it induces apoptosis of cancer cells. However, there are fewer reports on the effect of cryptotanshinone on autophagy in human OSCC cell lines. In this study, we examined the effects of cryptotanshinone on the growth and chemosensitivity of HN-6 and HSC-3 cells *in vitro* and *in vivo*. In addition, a series of test assays were also performed to investigate whether cryptotanshinone exerts anticancer effects by regulating autophagy pathways.

Materials and methods

Cell culture and treatment. Human OSCC cell lines, HSC-3 and HN-6 were obtained from the Laboratory of Stomatological Hospital Affiliated to Chongqing Medical University (Chongqing, China). The cell lines were authenticated by short tandem repeat profiling and tested for mycoplasma contamination. These cells were cultured in DMEM-high glucose medium supplemented with 10% fetal bovine serum and penicillin and streptomycin at 37 °C in 5% CO_2 [23, 24].

Cell viability assay. Cells were cultured in a 96-well plate for 24 h and then treated with cryptotanshinone at different concentrations. The cell viability was determined by the Cell Counting Kit-8 (CCK-8) according to the manufacturer's guidelines, and the absorbance was quantified by an automated microplate spectrophotometer at 450 nm.

Annexin V-FITC staining assay. Cell apoptosis was determined using an Annexin V-FITC Apoptosis Detection Kit [25]. In brief, cells were suspended in a binding buffer and stained with Annexin V-FITC for 15 min at room temperature in darkness. Apoptosis was assessed and analyzed using a C6 flow cytometer.

Transmission electron microscopy (TEM). After OSCC cells were treated with the corresponding experimental design described above, TEM was used to observe autophagosome in OSCC cells. After cells were washed with phosphate buffer saline (PBS), they were digested with 0.25% trypsin and centrifugation in a 1.5 ml tube. Then sediment was fixed with glutaraldehyde for 2 h at 4 °C. Subsequently, samples were post-fixed with osmium and dehydrated with ethanol. Finally, samples were examined via TEM [26].

Immunofluorescence staining. The cells were fixed with 4% paraformaldehyde for 10 min and blocked with 10% goat serum for 2 h after being permeabilized with 0.1% TritonX-100 for 10 min. After washing with 1% TBST three times, cells were incubated with the primary antibody against LC3B (1 μg/ml, Abcam, ab192890) at 4 °C overnight, and then incubated with the appropriate fluorescent secondary antibody at room temperature for 2 h. The cells were counterstained with 4`,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, 62248), and the images were visualized by laser scanning confocal microscopy [27].

Western blot analysis. Cells were treated with different concentrations of cryptotanshinone (10–20 μM) for 36 h at 37 °C. The cell lysates were then harvested and resolved by SDS/PAGE. After electrophoresis, the proteins from SDS/PAGE were electrotransferred to a membrane, which was then blocked with 5% dried milk for 60 min. The membrane was then washed three times for 5 min with TBST wash buffer and immunoblotted with the appropriate antibodies overnight at 4 °C. The membrane was then incubated with HRP-conjugated secondary antibodies for 60 min. Band intensities were quantified with ImageJ (NIH) [28]. The antibodies used included β-actin (1:1000, Abcam, ab8226), Beclin 1 (1:1000, Abcam, ab207612), LC3B (1:1000, Abcam, ab192890), Caspase-3 (1:1000, Abcam, ab32351), Cleaved Caspase-3 (1:500, Abcam, ab32042), ATG9A (1:1000, Abcam, ab108338), ATG5 (1:1000, Abcam, ab108327), ATG4B (1:1000, Abcam, ab154843). The reaction was visualized using Clarity Western ECL substrate and detected by exposure to autoradiographic film.

Tumor xenograft experiment. Tumor xenograft experiments were performed as described previously [29, 30]. The male nude mice aged 6–8 weeks were maintained in standard conditions and cared for according to the institutional guidelines for animal care. HSC-3 cells and HN-6 cells in PBS and Matrigel with equal volume were subcutaneously injected into mice to establish tumor xenografts. When the diameter of the tumor reached about 5 mm [31], the mice were randomly divided into two groups (n=6 mice/group) and treated with cryptotanshinone (30 mg/kg) by oral gavage, as well as vehicle as control, respectively, every 2 days. Tumor volumes were measured every 2 days in a blinded fashion, calculated by the formula of $V = (\text{length} \times \text{width}^2)/2$. At the end of the experiment, the tumors, livers, lungs, and kidneys were collected for Hematoxylin and Eosin (H&E) staining and immunohistochemical staining. All the animal experiments were approved by the Ethics Committee for Animal Experiments of the Stomatological Hospital of Chongqing Medical University.

Statistical analysis. All *in vitro* experiments were performed in three independent experiments. Data were expressed as the mean ± standard deviation (SD) and

compared by GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). Comparisons between the two groups were analyzed by a Student's t-test method. The comparisons among multiple groups were made with a one-way analysis of variance (ANOVA) followed by Tukey's test. A p-value <0.05 was considered significant.

Results

Cryptotanshinone induces apoptosis and decreases cell proliferation in HN-6 and HSC-3 cells. Cryptotanshinone (Figure 1A) was identified as a drug candidate inhibiting the proliferation of HN-6 and HSC-3 cells. To evaluate the effect of cryptotanshinone on the proliferation of HN-6 and HSC-3 cells, HN-6 and HSC-3 cells were treated with increasing concentrations of cryptotanshinone for 36 h. As shown (Figure 1B), cryptotanshinone inhibited cell proliferation in a dose-dependent manner. To determine the effect of cryptotanshinone on the apoptosis of HN-6 and HSC-3 cells, cells were treated with the indicated concentrations (up to 80 μ M) of cryptotanshinone for 36 h, and the results (Figure 1C) showed that cryptotanshinone induced apoptosis in a dose-dependent manner and caused apoptosis in HN-6 and HSC-3 cells. After the cells were treated with cryptotanshinone at different concentrations, the expression of Caspase-3 and Cleaved Caspase-3, the markers of apoptosis,

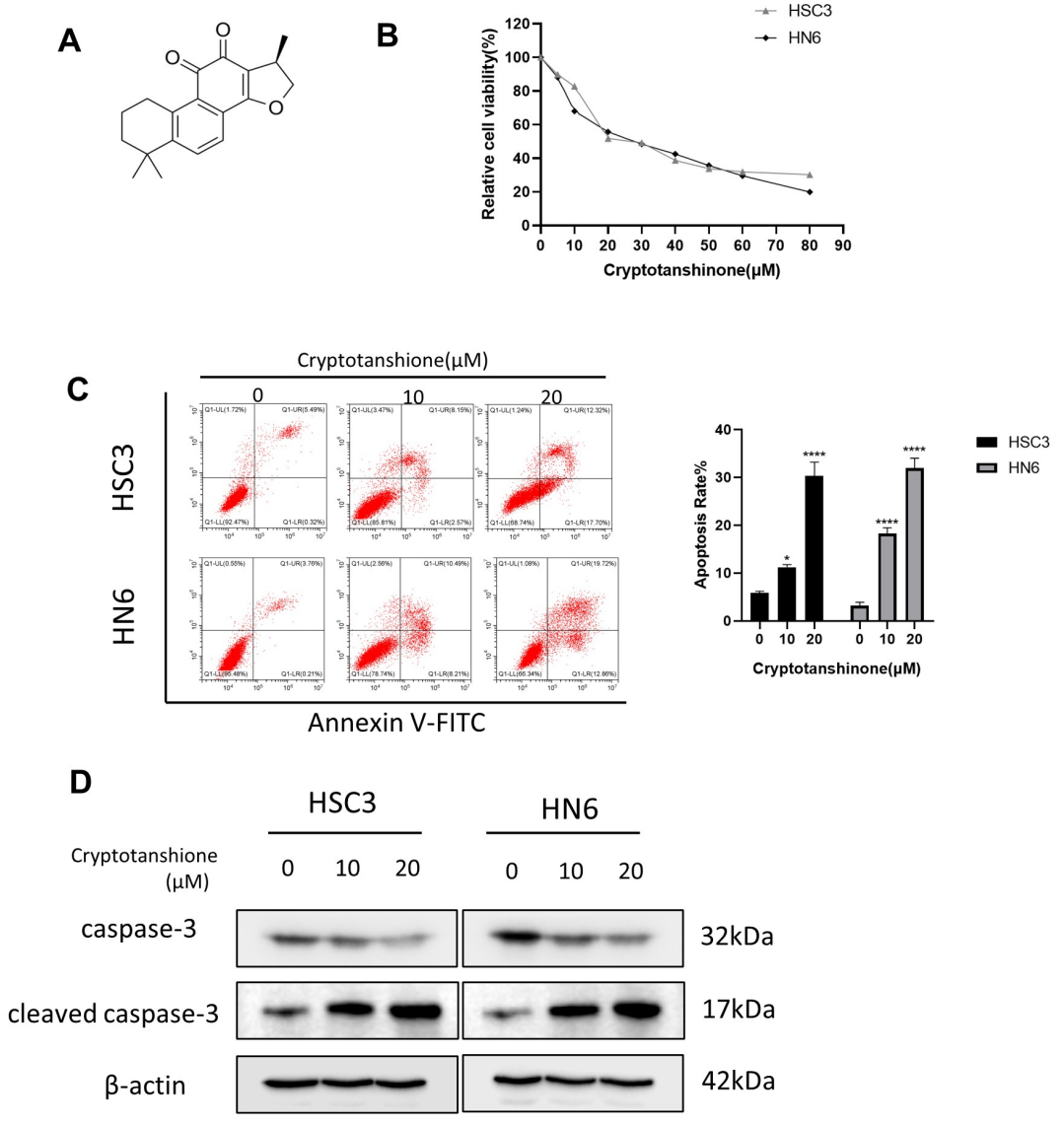

Figure 1. Cryptotanshinone inhibits OSCC cell proliferation. A) Chemical structure of echinatin. B) HSC3 and HN6 cells were treated with cryptotanshinone at different concentrations (for up to 80 μM and 36 h), and cell viability was determined by CCK-8 assay. C) The detection of apoptosis in OSCC cells treated with different concentrations of cryptotanshinone for 36 h by an Annexin V-FITC staining assay. D) Western blot analysis was performed to detect the expression of Caspase-3 and Cleaved Caspase-3 in the OSCC cells treated with indicated concentrations of cryptotanshinone for 36 h. Bars, SD.

changed. This result further confirmed that cryptotanshinone dose-dependently induced apoptosis (Figure 1D). Taken together, these data suggest that cryptotanshinone can trigger apoptosis to inhibit the proliferation of HN-6 and HSC-3 cells.

Cryptotanshinone induces autophagy in HN-6 and HSC-3 cells. We next examined whether cryptotanshinone could induce autophagy in HN-6 and HSC-3 cells. The presence of autophagosomes was observed in cells subjected to cryptotanshinone treatment as visualized by electron

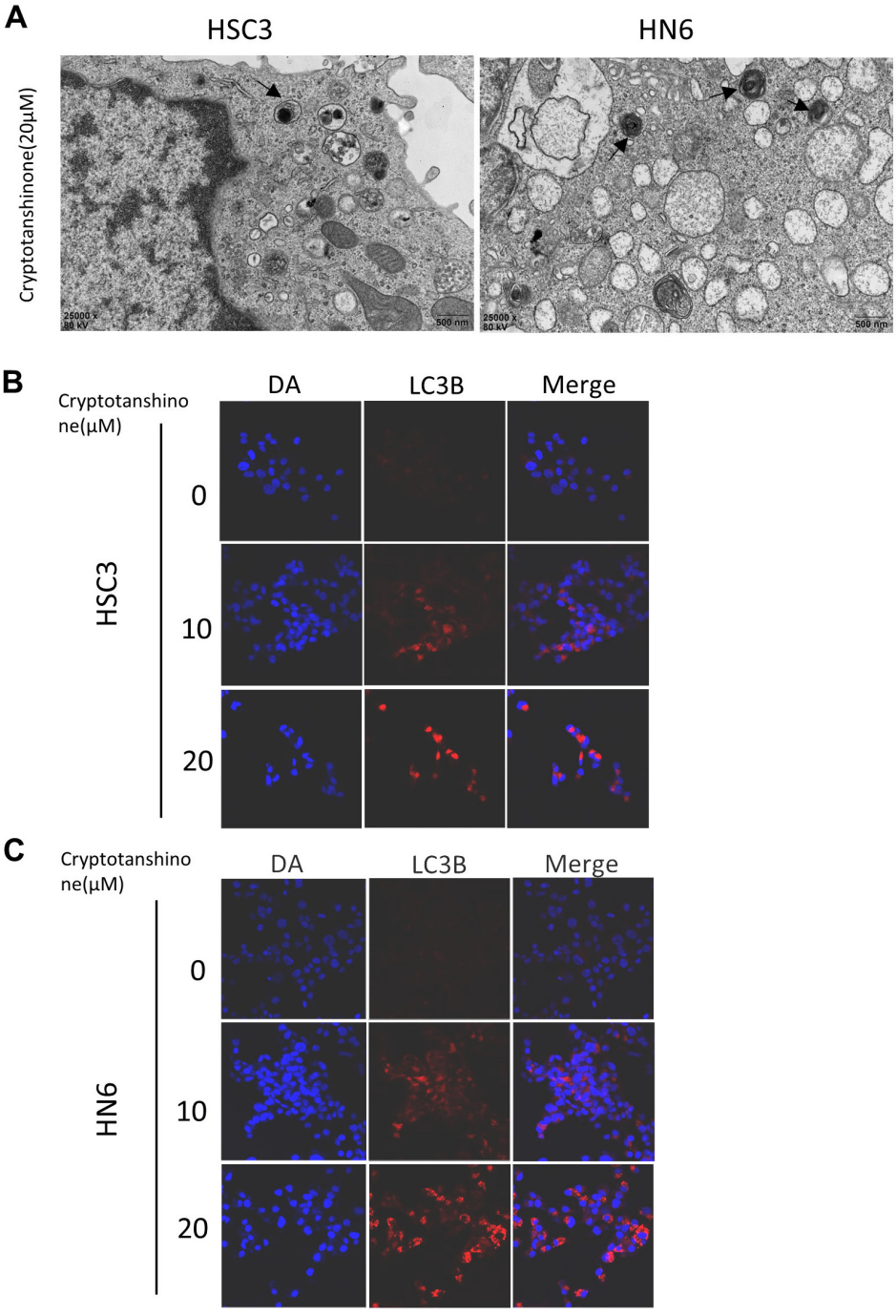

Figure 2. Cryptotanshinone induces autophagy in OSCC cells. A) Observed under an electron microscope, exposed to cryptotanshinone (20 μM) HSC-3 and HN-6 cells to produce autophagosomes. B, C) The expression of LC3B in the HSC-3 and HN-6 cells exposed to cryptotanshinone at different concentrations was compared by immunofluorescence. GraphPad Prism 8.0 was used to process the image and to compare the overall fluorescence intensity of LC3B spots.

Table 1. Comparison of serum ALT and AST level between cryptotanshinone-treated and control groups.

Group Name	Serum ALT activity (U/l)			Serum AST activity (U/l)		
HSC3	43.261	35.724	32.357	147.961	126.492	80.189
HSC3 (30 mg/kg)	84.276	49.569	65.324	136.256	119.657	94.319
HN6	31.598	43.568	56.265	130.946	109.351	95.184
HN6 (30 mg/kg)	46.254	36.697	41.268	113.512	123.789	93.254

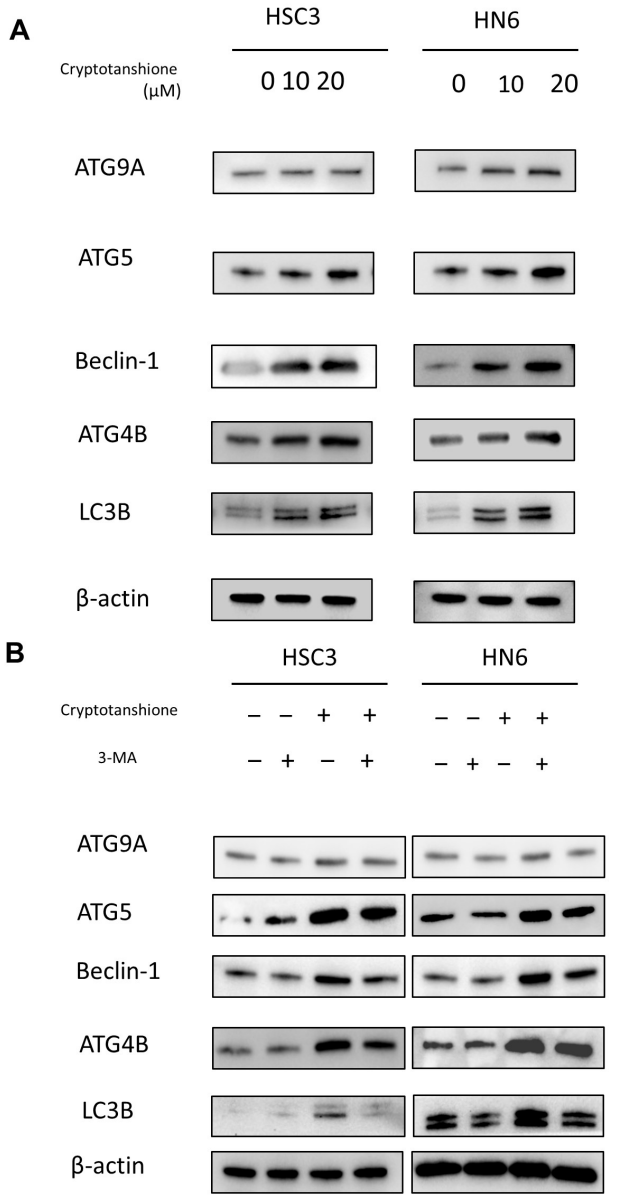

Figure 3. Different concentrations of cryptotanshinone affect the expression of autophagy-related proteins in OSCC cells. A) HSC-3 and HN-6 cells were exposed to different concentrations of cryptotanshinone for 36 h, and western blot analysis was performed to detect the expression levels of ATG9A, ATG5, ATG4B, Beclin-1, and LC3B. B) Comparison of expression of autophagy markers in OSCC cells treated with different concentrations of cryptotanshinone with or without 3-MA pretreatment (50 μM, 36 h) by western blot.

microscopy (Figure 2A), demonstrating that cryptotanshinone can activate an autophagic response within cells. The expression of LC3B is closely related to the level of autophagy. Confocal microscopy analysis demonstrated that exposure of HN-6 and HSC-3 cells to cryptotanshinone resulted in the accumulation of cellular LC3B puncta (Figures 2B, 2C), suggesting an increase in autophagy levels within the cells.

Furthermore, western blot results confirmed increased LC3B, Atg9A, Atg5, Atg4B, and Beclin 1 expression in cryptotanshinone-treated cells in a dose-dependent manner (Figure 3A). In addition, cryptotanshinone-induced cell autophagy was reversed after pretreatment with the autophagy inhibitor 3-MA as shown in Figure 3B [32]. These results suggest that cryptotanshinone may induce apoptosis in HN-6 and HSC-3 cells by activating autophagy.

Cryptotanshinone inhibits the growth of HN-6 and HSC-3 tumor xenografts *in vivo*. Nude mice bearing HN-6 and HSC-3 derived tumor xenografts were orally administered with cryptotanshinone (30 mg/kg), and its effect on tumor growth was monitored. In the group receiving 30 mg/kg cryptotanshinone, tumor burden was significantly suppressed, with reductions of 51% and 58% (Figures 4A, 4C), respectively. In terms of body weight, there was no significant difference between the treatment and control groups (Figure 4B). Hematoxylin and eosin staining showed that after the administration of drugs, the tumor body cells appear the phenomenon of increased necrosis (Figure 4D). After subjecting the sections to immunohistochemical staining experiments for LC3B protein, the phenomenon of increased LC3B puncta was found (Figure 4E), indicating that autophagy was triggered by cryptotanshinone increasing the LC3B expression level in tumor cells.

In addition, cryptotanshinone treatment did not induce any obvious changes in the morphology of vital organs, including the lung, liver, and kidney (Figure 4F). In addition, no significant differences in the serum levels of alanine transaminase (ALT) or aspartate transaminase (AST) in nude mice were observed (Table 1), indicating that cryptotanshinone had no toxic effects on the animals.

Discussion

Autophagy is a highly evolutionarily conserved mechanism best known for its role in organelle and protein turnover, cellular quality control, and metabolism, in which cytoplasm, organelles, and proteins are sequestered into autophagosomes that then fuse with lysosomes for degeneration to maintain cell turnover and homeostasis during starvation, stress, microbial invasion, and inflammation. Dysregulation of autophagy is closely associated with many diseases, including cancer, cardiovascular and autoimmune diseases [33–35].

Many stimuli leading to apoptosis can trigger autophagy, which usually appears before apoptosis. Autophagy activation beyond a certain threshold may lead to the collapse of

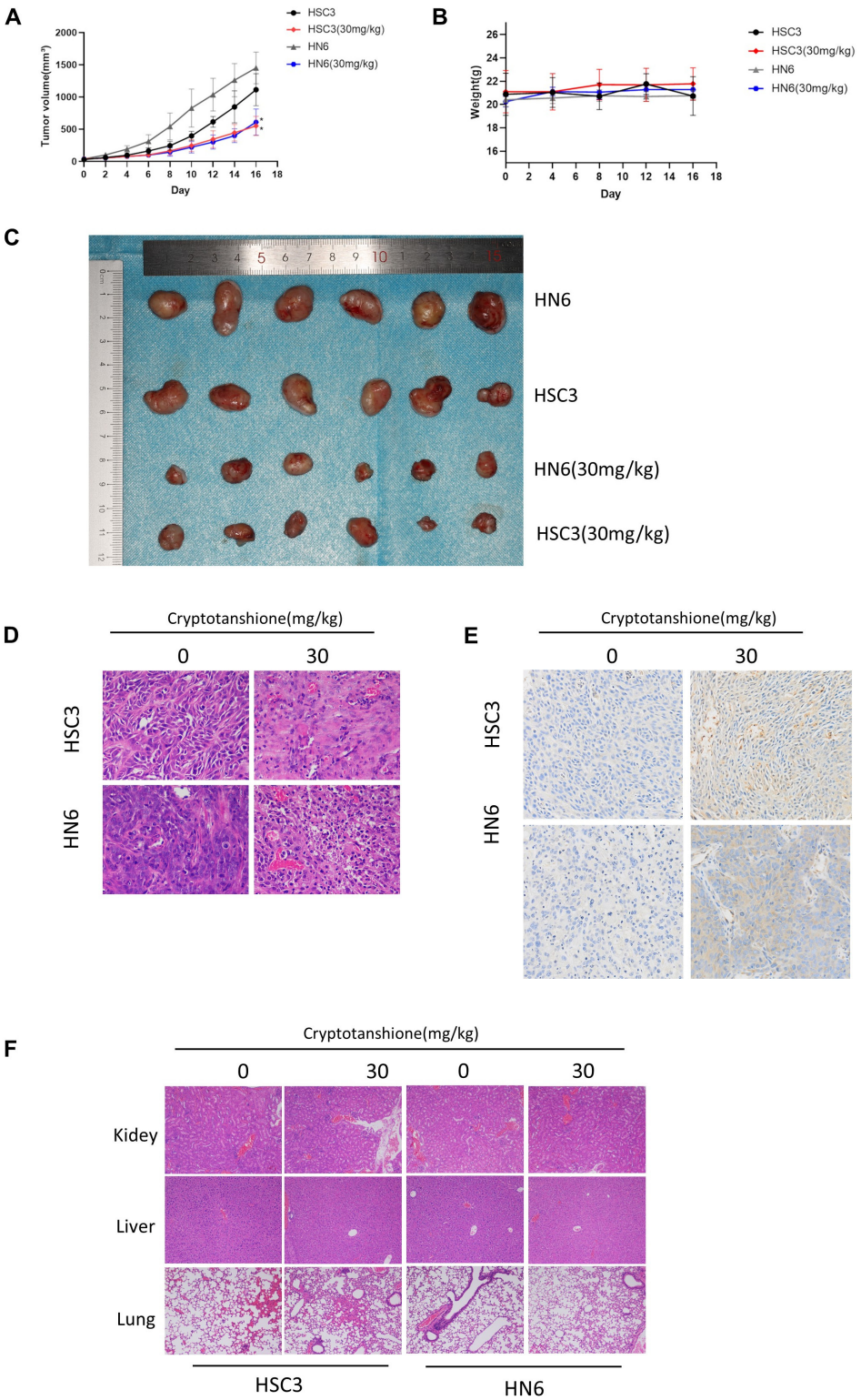

Figure 4. Cryptotanshinone suppresses the growth of OSCC tumor xenograft in nude mice. The nude mice bearing HSC3-derived and HN6-derived tumor xenografts were orally administrated with cryptotanshinone (30 mg/kg) every 2 days (n=6/group), whereas the control group received the vehicle only. A) Tumor curves showed that cryptotanshinone significantly suppressed the growth of tumor xenografts. B) Body weight of nude mice during the experimental period. C) Image of removed xenograft tumors at 16 days. D) H&E staining of tumor xenografts collected from mice of the treatment and control groups. E) Immunohistochemical staining was performed on the sections, in the treatment group brownish-yellow LC3B puncta increased. F) H&E staining of kidney, liver, and lung specimens collected from mice of the treatment and control groups. Bars, SD; *p<0.05

its function, resulting in autophagic cell death or other types of cell death. Studies related to genetic experiments have also reported that autophagy may play an important role in the control of apoptosis, and as such, it is well-recognized that autophagy can induce cell death to suppress cancer progression and may therefore be a target for cancer therapy [36–39].

In this study, we found that cryptotanshinone increased the expression level of autophagy marker protein LC3B and other autophagy-related proteins, indicating that cryptotanshinone can promote the expression of autophagy-related proteins in cells, that is to say, cryptotanshinone promotes autophagy. In addition, when HN-6 and HSC-3 cells were pretreated with autophagy inhibitor 3-MA, the effect of cryptotanshinone on autophagy-related proteins was significantly weakened, which indicated that cryptotanshinone was very relevant to autophagy activation in HN-6 and HSC-3 cells. Emerging evidence suggests that the autophagy pathway can activate a range of processes involved in tumor initiation and progression, including cell proliferation, differentiation, survival, chemoresistance, and metastasis, and thus may be a potential target for anticancer therapy [40–42].

Epidemiological and experimental data clearly demonstrate a link between inflammation, fibrosis and [43–46], for example, inflammatory diseases such as heavy aphthous ulcers and Behcet's have been associated with an increased risk of oral cancer [47, 48]. Anti-inflammatory therapy shows great efficacy in cancer prevention and treatment [49, 50], this adds to the anti-cancer bioactivity research of anti-inflammatory drugs. In fact, cryptotanshinone was originally described as a Danshen extract with anti-fibrotic and anti-inflammatory activities, and our findings confirm that it also has anticancer effects. Moreover, whether the anticancer bioactivity of cryptotanshinone is related to its antifibrotic and anti-inflammatory properties deserves further investigation. Natural products such as traditional Chinese medicine have long been used to treat cancer. Given their multi-component and multi-target characteristics in pharmacology, natural products are a promising resource for screening anticancer drugs. Curcumin and resveratrol are classical examples of plant-derived compounds, and more new generations of drugs have been developed and applied in the clinic [51, 52]. Cryptotanshinone is the active component of *Salvia miltiorrhiza*, which has various biological activities. Although the chemical properties of Danshen have been extensively studied, the active components that contribute to its biological functions remain unclear. Tanshinone IIA, another diterpene isolated from licorice, has been reported to inhibit cell viability in lung cancer and oral squamous cell carcinoma [53–55]. In this study, our results showed that cryptotanshinone decreased cell proliferation and colony formation in a dose-dependent manner and, more importantly, inhibited the growth of HN-6 and HSC-3 tumor xenografts *in vivo*, whereas no damage was observed to normal cells or vital organs.

In conclusion, we identified cryptotanshinone as a potential anticancer natural product with inhibitory effects on HN-6 and HSC-3 tumorigenesis and growth potential. Mechanistically, cryptotanshinone can induce autophagy and apoptosis of HN-6 and HSC-3 cells by affecting the expression of autophagy-related proteins. This is the forceful demonstration that cryptotanshinone inhibits oral tumor cell proliferation through the autophagy pathway. These findings provide conclusive evidence for the potential of cryptotanshinone as a therapeutic option for the treatment of HN-6 and HSC-3 cells.

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