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ORIGINAL RESEARCH

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Study on the chemotherapeutic effect and mechanism of cucurbitacin E on laryngeal cancer stem cells

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Abstract

Objectives: Study on the chemotherapeutic effect and mechanism of cucurbitacin E (CuE) on laryngeal cancer stem cells.

Methods: We used flow cytometry to sort out $CD133^{+}$ laryngeal cancer stem cells; trypan blue rejection assay to detect the survival rate of laryngeal cancer stem cells; Cell counting kit-8 (CCK-8) assay to detect the effect of CuE on the proliferation ability of stem cells and the chemotherapeutic potentiation of doxorubicin; Transwell assay to observe the effect of CuE on the migration ability of stem cells; and Western Blot to detect the effect of CuE on the expression level of stem cell-associated proteins. The tumor volume of nude mice was measured at the end of the experiment, and paraffin sections of nude mice tumor tissues were prepared and stained with Hematoxylin and eosin (H&E). The expression of c-MYC in tumor tissues of nude mice was further detected by immunohistochemistry, and the effect of CuE on the expression level of related proteins in tumor tissues of nude mice was detected by Western Blot.

Results: CuE reduced the survival rate, proliferation ability, and migration ability of laryngeal cancer stem cells in vitro, and that CuE had a chemotherapeutic potentiating effect on doxorubicin. The possible mechanism of the chemotherapeutic effect of CuE was to reduce the expression of c-MYC protein, and the possible mechanism of chemotherapy synergy was to reduce the expression of ABCG2 and P-gp protein.

Conclusion: CuE has a chemotherapeutic effect on laryngeal cancer stem cells, as well as a chemotherapy synergy.

KEYWORDS
ABCG2, P-gp, c-MYC, cucurbitacin E, laryngeal cancer stem cells, CD133

1 | INTRODUCTION

Cancer stem cells (CSC) are a small subpopulation of cancer cells with self-renewal potential, capable of maintaining tumor growth and cell differentiation, involved in metastatic process, recurrence, and

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resistance to chemotherapeutic agents,[1] and may be the main source of cancer invasion, migration and extensive metastasis,[2,3] laryngeal squamous cell carcinoma in the second rank of head and neck squamous cell carcinoma; tumor stem cells (CSC) play a key role in the development and progression of head and neck squamous cell carcinoma (HNSCC), and can result tumor growth and malignant behavior.[4] Laryngeal cancer stem cells can be isolated from laryngeal squamous cell carcinoma tissue, and laryngeal cancer stem cells can be used to study the occurrence, development, and treatment strategies of laryngeal cancer, the most common CSCs markers are CD44, CD24, CD133, and ALDH1A1.[5] Previous studies by our group have demonstrated that CD133 is one of the markers of tumor initiating cells in the human laryngeal cancer Hep-2 cell line,[6] CD133 positive cells have a significant ability to generate new tumors in vivo[7] with stemness phenotype characteristics,[8] and CD133+ laryngeal cancer stem cells have stronger invasive, migratory, and tumorigenic abilities compared with normal laryngeal cancer stem cells.[9] Cucurbitacin E (CuE), an active compound of the cucurbitacin family, is a highly oxidized steroid consisting of tetracyclic triterpenes, which are more promising anticancer triterpenes[10,11] with multiple pharmacological functions and chemotherapeutic potential. CuE has inhibitory effects on various cancers,[12,13] such as CuE can inhibit the growth and invasion of osteosarcoma and esophageal cancer by inhibiting PI3K/Akt/mTOR signaling pathway,[14,15] and CuE has also been identified as an inhibitor of ABCB5 transporter protein in drug-resistant tumor cell lines.[16] However, the role and mechanism of CuE in laryngeal cancer stem cell chemotherapy is poorly studied. In this study, we intend to investigate the role of CuE in laryngeal cancer stem cell chemotherapy and the related mechanisms through in vivo and in vitro experiments.

2 | MATERIALS AND METHODS

Human laryngeal cancer cell line Hep-2 purchased from Qi Biotechnology Co.; fetal bovine serum (FBS) purchased from ExCell Co.; CuE purchased from Macklin Co.; RPMI1640 medium purchased from Hyclone Co.; CD133 antibody purchased from Immunoway Co.; ABCG2 rabbit anti-human monoclonal antibody purchased from Immunoway Co.; P-gp mouse anti-human monoclonal antibody purchased from Immunoway Co.; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) purchased from Wuhan Seville Biotechnology Co., Ltd.; sheep anti-mouse secondary antibody purchased from Wuhan Seville Biotechnology Co., Ltd.; penicillin and streptomycin purchased from Macklin Co.; basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and B27 purchased from Beijing BoaoSen Biotechnology Co., Ltd.; c-MYC antibody purchased from Wuhan Seville Biotechnology Co., Ltd.; 20 nude mice, mass (15 ± 2) g, were purchased from Hunan Sja laboratory Animal Co., Ltd, license No. SCXK (Xiang) 2019-0004, SPF grade, housed in four cages, five rats/cage, at the Experimental Animal Center of Lanzhou University. All animal studies and experimental procedures were approved by the Animal Care and Use Committee of the animal facility at Lanzhou University.

2.1 | Sorting and culturing of laryngeal cancer stem cells

The laryngeal cancer cell line Hep-2 was purchased from Qi Biotechnology Co. The cells were cultured in RPMI-1640 complete medium containing 10% FBS at 37°C in a 5% CO_2 saturated humidity incubator, and the cells were passaged when they grew to 80% densities. In this experiment, $CD133^+$ tumor stem cells were sorted by flow cytometry, and the sorted laryngeal cancer stem cells were cultured in suspension using ultra-low suspension six-well plates with DMEM/F12 medium, and serum-free additives such as 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml bFGF, 20 ng/ml EGF, and 2% B27 were added to the medium.

2.2 | Detection of cell viability by trypan blue exclusion

Cells were collected from each group after 24 h of the action of different concentrations of CuE. Single cell suspensions were prepared by trypsin digestion and mixed with 0.4% trypan blue solution at 9:1 for 3 min, and blue-stained and non-stained cells were counted under an inverted microscope at ×100, and the cell survival rate was calculated [cell survival rate = total number of live cells/(total number of live cells + total number of dead cells) × 100%].

2.3 | Effect of CuE on the proliferative capacity of stem cells by cell counting kit-8 assay

The effect of CuE on the proliferation of stem cells was examined by Cell counting kit-8 (CCK-8) method. In 96-well plates, 10,000 cells were seeded in each well with different concentrations of CuE (0, 12.5, 25, 50, and 75 μmol/L), and three replicate wells were set for each concentration. Then, 10 μl of CCK-8 was added after 4 h. The absorbance was measured at 12, 24, and 36 h after the action of different concentrations of CuE.

2.4 | Transwell assays to detect the effect of CuE on the migration ability of stem cells

Cells were treated with increasing concentrations of CuE (12.5, 25, 50, and 75 μmol/L) for 24 h. The cells were then dissolved in serum-free medium and seeded in the transwell migration plate at a density of (1000 cells/μl/chamber) and allow to grow for additional 12 h, and then fixed with formaldehyde at room temperature for 0.5 h and stained with crystal violet for 0.5 h. The cells in the upper chamber that had not migrated were wiped with cotton swabs, photographed microscopically, and the number of cells passing through the membrane was counted in five random fields per membrane.

2.5 | Chemopotentiation of doxorubicin by cell counting kit-8 assay of CuE

To detect the chemotherapeutic potentiation effect of CuE on doxorubicin by CCK-8 method, 10,000 cells were seeded in each well of a 96-well plate and incubated with doxorubicin (10 μmol/L) and combination of doxorubicin and CuE (10 + 12.5, 10 + 25 μmol/L), 10 μl of CCK-8 was added after 4 h. The absorbance was measured at 12, 24, and 36 h after the action of different concentrations of drugs.

2.6 | Western Blot to detect the effect of CuE on the expression level of stem cell-associated proteins

Cells were grown to 80% density and incubated with different concentrations of CuE for 24 h. The proteins were extracted using RIPA, and the samples were separated by polyacrylamide gel electrophoresis, transferred to PVDF membrane by wet transfer method, and then closed with TBST containing skim milk powder at room temperature for 1 h. The primary antibody was covered with the membrane and incubated for 2 h. The horseradish peroxidase-labeled secondary antibody was added and incubated for 1 h at room temperature, The images were developed by the substrate chemiluminescence ECL method with GAPDH as the internal reference, and analyzed by ImageJ software.

2.7 | Cell line xenograft in nude mice

Five-week-old BALB/c-nu mice (male, 16–18 g, $n = 20$) were purchased from Hunan Sja laboratory Animal Co., Ltd, license number SCXK (Xiang) 2019-0004. Nude mice were housed in an environment free of specific pathogenic bacteria, with room temperature controlled at 22°C–25°C, humidity maintained at 50%–60%, and light/dark time of 12 h each, and fed ad libitum. The mice were acclimatized and fed for 1 week before the experiment. The nude mice were randomly divided into control group, doxorubicin concentration of 20 μmol/L group, CuE concentration of 75 μmol/L group, and doxorubicin and CuE combination (20 + 75 μmol/L) group, and Hep-2 cell density was adjusted to 5×10^6 cells/ml for subcutaneous injection. Chemotherapeutic drugs were started after 1 week of successful transplant tumor construction in nude mice. The mice were injected once every 2 days by peritumoral injection, and the dose of drug was 200 μl. The weight and tumor volume of mice were recorded every 2 days. After 30 days of drug action, the mice were executed, and some of the tumor masses were taken out and stored in −80°C refrigerator for paraffin-embedded sections for subsequent experiments.

2.8 | Hematoxylin and eosin staining and immunohistochemistry

At room temperature, the fixed tumor tissues were washed in a tissue box, placed in a dehydrator, dehydrated in gradient alcohol and transparent in xylene, dipped in wax, placed in an embedding frame, flattened at its bottom, and poured into liquid paraffin to make a paraffin tissue block. Paraffin tissue section, thickness of 5 μm. sections were unfolded in warm water at 53°C, flattened and attached to slides, baked at 60°C baking machine for 3 h, dewaxed, and hydrated, hematoxylin staining, differentiated anti-blue treatment, eosin staining, PBS water washing, dehydrated, and transparently sealed, and the results were observed by light microscope.

Paraffin blocks of tumor tissues from different groups of nude mice were serially sectioned, dewaxed in xylene, rehydrated, antigen repair using EDTA with PH 9.0 for 20 min; PBS rinsed and added 3% H_2O_2 dropwise for 10 min; PBS rinsed and added 100 μl of c-MYC antibody dropwise, respectively, incubated at 37°C for 60 min, PBS rinsed and added 100 μl of enzyme-labeled goat anti-mouse/rabbit IgG polymer, incubated at room temperature for 15 min, PBS rinsed and then DAB color development, hematoxylin re-stained, xylene transparent, neutral gum sealed, light microscope observation of the results, and immunohistochemical semi-quantitative analysis using Image pro plus software.

2.9 | Detection of ABCG2 and P-gp protein relative expression in tumor tissues of nude mice by Western Blot

Cell lysate RIPA was added to each group of tumor tissues of about 5 × 3 mm in size, placed on ice and lysed for 30 min; tumor tissues

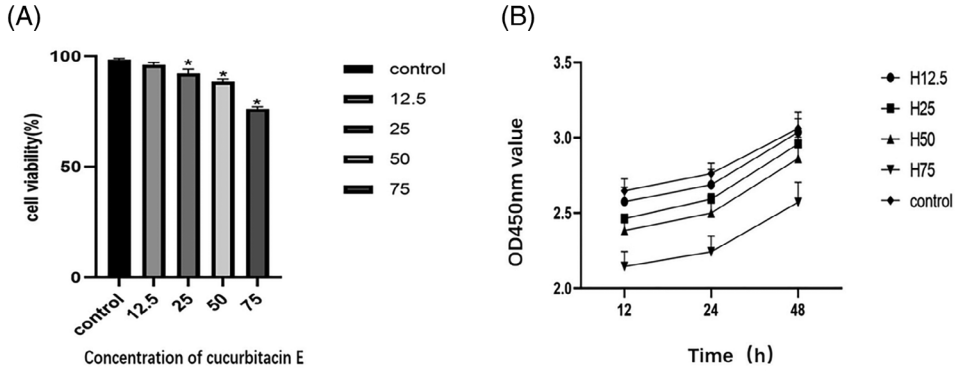

FIGURE 1 (A) Effect of cucurbitacin E on the survival rate of laryngeal cancer stem cells using different concentrations of cucurbitacin E. Statistical analysis *$P < .05$ versus control group. (B) Effect of cucurbitacin E on the proliferative capacity of laryngeal cancer stem cells

were ground and aspirated 1.5 ml in EP tubes, centrifuged at 4°C and 12,000 r/min for 5 min, aspirated 1.5 ml supernatant; protein concentration in tumor tissues was determined by BCA method; gel was prepared according to SDS/PAGE kit; and electrophoresis was performed. The protein was transferred to PVDF membrane by wet transfer method, closed at room temperature for 2 h, added primary antibody dilution ABCG2, P-gp (concentration 1:1000), incubated overnight at 4°C, washed with TBST, added HRP-labeled goat anti-rabbit secondary antibody, incubated at room temperature for 1 h, developed by substrate chemiluminescence ECL method with GAPDH as internal reference, and the images were analyzed with the help of ImageJ software. The images were analyzed by ImageJ software.

Statistical analyses were performed using SPSS 25.0 (IBM, Armonk, NY) and GraphPad Prism 8.0 (GraphPad, San Diego, CA). Data were expressed as mean ± standard deviation. One-way analysis of variance was utilized to analyze the differences between groups, and differences were considered statistically significant at $P < .05$.

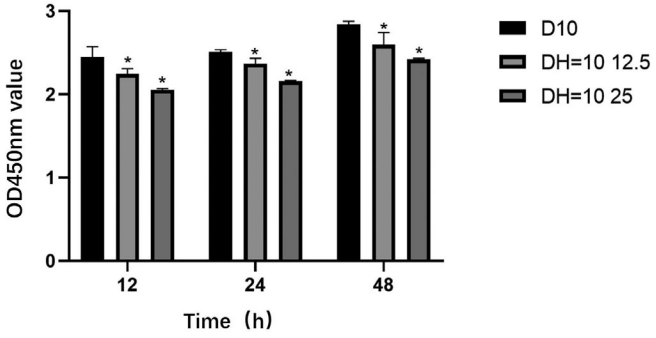

FIGURE 2 Comparison of the ability of doxorubicin alone and doxorubicin in combination with different concentrations of cucurbitacin E to inhibit the proliferation of laryngeal cancer cells, statistical analysis *$P < .05$ versus control group

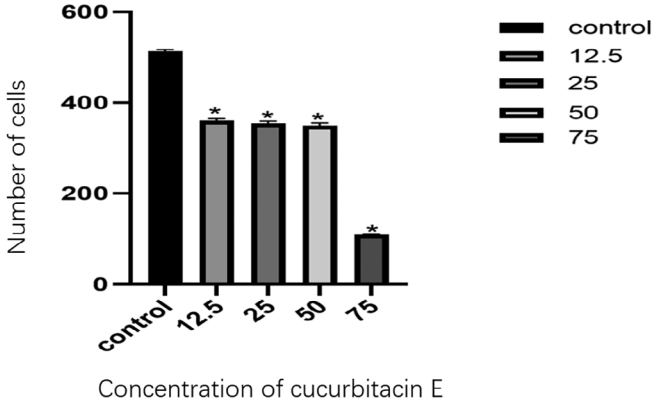

FIGURE 3 The effect of cucurbitacin E on the migration ability of laryngeal cancer stem cells was examined using different concentrations of cucurbitacin E. Statistical analysis *$P < .05$ versus control group

3 | RESULTS

3.1 | Cell viability by trypan blue rejection assay

In the experiment, different concentrations of CuE were used to act on laryngeal cancer stem cells, and the survival rate of laryngeal cancer stem cells was calculated by staining different groups of laryngeal cancer stem cells with trypan blue. The difference in survival rate between CuE and control group was statistically significant, as shown in Figure 1.

3.2 | Effect of CuE on the proliferation ability of laryngeal cancer stem cells by cell counting kit-8 assay

The results showed that the proliferation activity of stem cells in vitro was significantly inhibited by CuE at a concentration of 12.5 μmol/L, which was enhanced with the increase of CuE concentration and the cell proliferation ability decreased (Figure 1B).

3.3 | Chemopotentiation of doxorubicin by cell counting kit-8 assay of CuE

Experiments with doxorubicin (10 μmol/L) and doxorubicin in combination with different concentrations of CuE (10 + 12.5, 10 + 25 μmol/L)

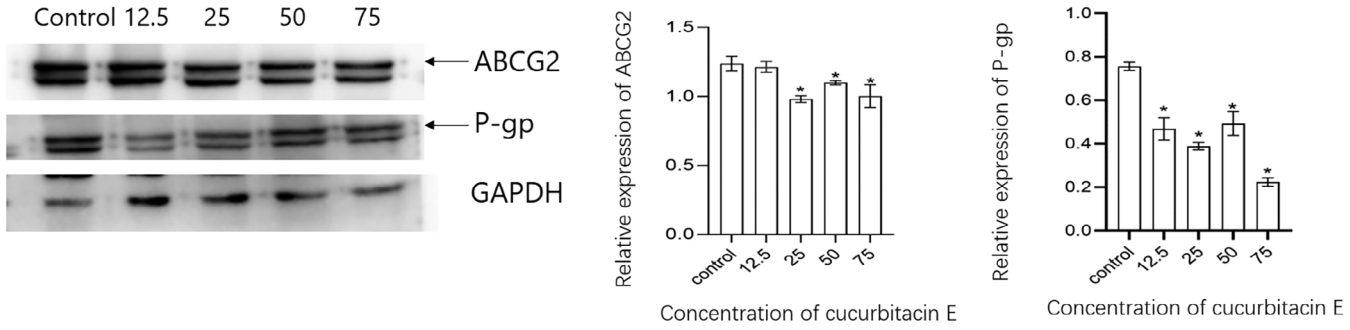

FIGURE 4 The effect of different concentrations of cucurbitacin E on the expression of ABCG2 and P-gp protein in laryngeal cancer stem cells was examined by statistical analysis *$P < .05$ versus control group

showed that doxorubicin in combination with CuE inhibited cell proliferation more significantly than doxorubicin alone, as shown in Figure 2.

3.4 | Transwell assay to detect the effect of CuE on the migration ability of laryngeal cancer stem cells

It was found that compared with the control group, each experimental group could significantly inhibit the migration ability of laryngeal cancer stem cells, and the difference was statistically significant; however, the migration ability of laryngeal cancer stem cells was not inhibited in a dose-dependent effect, and the migration ability of laryngeal cancer stem cells was most strongly inhibited when the concentration of CuE was 75 μmol/L, as shown in Figure 3.

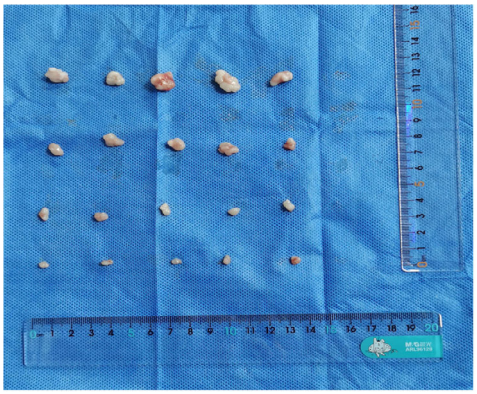

FIGURE 5 Photographs of tumor tissue in nude mice. From top to bottom, the control group, doxorubicin group, cucurbitacin E group, and doxorubicin and cucurbitacin E combination group

3.5 | Western Blot detection of the effect of CuE on the expression level of stem cell-associated proteins

It was found that there was no statistical difference in the expression of CuE at a concentration of 12.5 μmol/L compared with the control group to inhibit the expression of the drug-resistant protein ABCG2, whereas the expression of the drug-resistant protein ABCG2 could be inhibited when the concentration of CuE was 25, 50, and 75 μmol/L; and each experimental group could inhibit the expression of the drug-resistant protein P-gp to different degrees compared with the control group, and the differences were statistically significant (Figure 4).

3.6 | Measurement of tumor volume in nude mice

At the end of the experiment, the tumor volumes of nude mice were control group (1108.592 ± 266.3343) mm^3, doxorubicin group (445.0467 ± 88.94465) mm^3, CuE group (338.962 ± 51.62946) mm^3, and combined doxorubicin and CuE group (174.5664 ± 87.54595) mm^3, respectively. Compared with the control group, all experimental groups significantly inhibited the growth of tumors in nude mice, and the combined drug group inhibited the growth of tumors in nude mice most significantly (Figure 5).

3.7 | Hematoxylin and eosin staining of nude mouse tumor tissue

In the experiment, Hematoxylin and eosin (H&E) staining of tumor tissues showed that the tumor cells in the control group had large

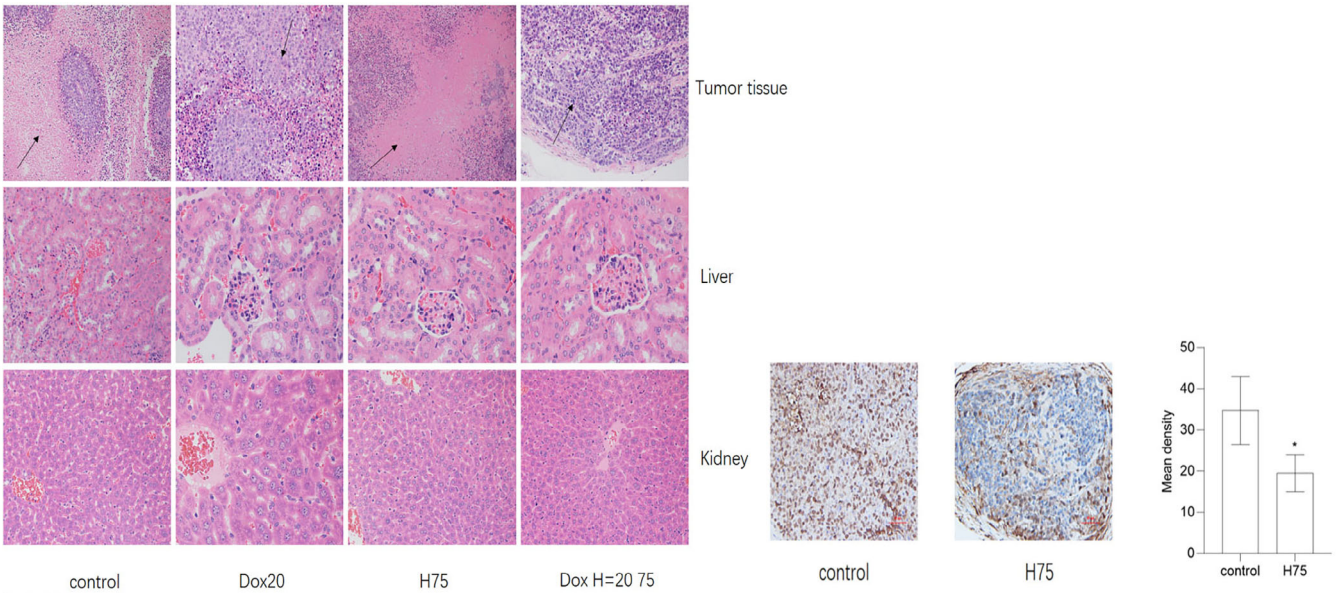

FIGURE 6 H&E staining of tumor tissues, kidney, and liver of nude mice using cucurbitacin E, doxorubicin, and the combination of both drugs. Immunohistochemical detection of c-myc expression in the tumor tissues of control and nude mice after the effect of cucurbitacin E concentration of 75 μmol/L and semi-quantitative analysis of the results, statistical analysis *$P < .05$ versus control

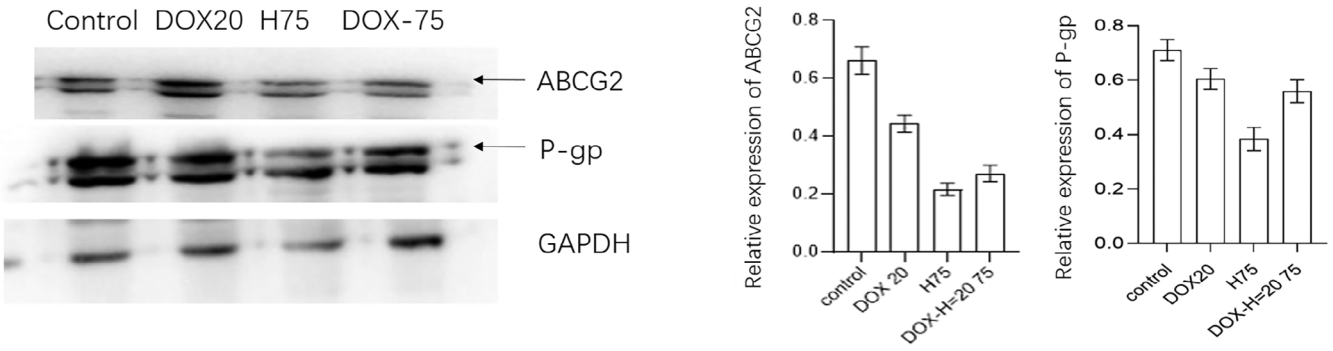

FIGURE 7 The effects of cucurbitacin E, doxorubicin, and their combination on the tumor tissues of nude mice were detected by measuring the expression of ABCG2 and P-gp protein in the tumor tissues

heterogeneity and pathological nuclear schizophrenia, whereas the cancer cells in each experimental group showed different degrees of degenerative changes compared with the control group, and a large number of lymphocytes, neutrophils, and phagocytes were infiltrated, and fibrous tissue proliferation in the tumor bed was seen locally. The H&E staining of liver and kidney of nude mice did not show any abnormalities (Figure 6).

3.8 | Immunohistochemical detection of c-MYC expression in tumor tissues of nude mice

The tumor tissues of control nude mice and the tumor tissues of nude mice after peritumor injection of CuE at a concentration of 75 μmol/L were subjected to immunohistochemical assays, and the c-MYC protein expression was analyzed semi-quantitatively using Image pro plus software, and it was found that CuE at a concentration of 75 μmol/L significantly reduced the expression of c-MYC protein in tumor tissues of nude mice compared with the control group (Figure 6).

3.9 | Effect of CuE on the expression level of tumor-related proteins in nude mice by Western Blot

The expression of ABCG2 and P-gp protein was detected by Western Blot in the control group and each experimental group of nude mice tissues, and it was found that each experimental group could significantly reduce the expression of ABCG2 and P-gp protein, and the difference was statistically significant, and the use of CuE (75 μmol/L) alone reduced the expression of ABCG2 and P-gp protein more significantly, as shown in Figure 7.

4 | DISCUSSION

In the CCK-8 assay, the concentrations of CuE were set at 0, 12.5, 25, 50, and 75 μmol/L. It was found that CuE inhibited the proliferation of laryngeal cancer stem cells in a dose-dependent manner, but even the concentration of CuE at 75 μmol/L did not reach the half inhibition concentration to laryngeal cancer stem cells. Trypan blue exclusion assay showed that CuE inhibited the viability of laryngeal cancer stem cells in a dose-dependent manner. However, when the effect of CuE on the migration ability of laryngeal cancer stem cells was examined by Transwell assay, there was no obvious dose-dependent effect on the migration ability of laryngeal cancer stem cells, and the migration ability of laryngeal cancer stem cells was most inhibited when the concentration of CuE was 75 μmol/L. This may be related to the fact that CuE can also play a stronger tumor-killing effect at this concentration. The combined effect of CuE could kill laryngeal cancer stem cells and inhibit the migration ability of laryngeal cancer stem cells, which resulted in a significant decrease in the number of membrane penetrating cells at a concentration of 75 μmol/L in the Transwell assay.

The development of multidrug resistance (MDR) to chemotherapy remains a major challenge in cancer therapy, and many mechanisms leading to MDR have been recognized, but one of the most important is the overexpression of adenosine triphosphate (ATP) binding cassette (ABC) transport proteins, a mechanism associated with the expression of proteins closely associated with MDR to chemotherapy, through which the efflux of various anticancer drugs is powered by ATP,[17,18] and studies have used the CCK-8 assay to detect the chemotherapeutic potentiation of doxorubicin by CuE. The CCK-8 method found that doxorubicin inhibited tumor cell proliferation at 10 μmol/L. Using doxorubicin (10 μmol/L) culture and doxorubicin in combination with different lower concentrations of CuE (10 + 12.5, 10 + 25 μmol/L), it was found that doxorubicin in combination with different lower concentrations of CuE inhibited cell proliferation more than doxorubicin alone. The effect of doxorubicin combined with different lower concentrations of CuE was found to be more significant than that of doxorubicin alone in inhibiting cell proliferation. The difference was statistically significant, and CuE may reduce the efflux of laryngeal cancer tumor stem cells to chemotherapeutic drug doxorubicin by decreasing the expression of ABC transporter ABCG2 and P-gp protein, so it exert chemotherapy potentiation effect, but this mechanism needs to be further elucidated by further experiments such as immune efflux assay.

In the tumorigenesis experiments in nude mice, considering that the drug did not act directly on the tumor cells of nude mice but on

the tumor tissues of nude mice, and that there might be some subcutaneous absorption or incomplete action on the tumor tissues during the injection, the concentrations of the drug used were the maximum concentrations of each group in the cellular experiments, and the peritumor injections of doxorubicin (20 μmol/L), CuE (75 μmol/L), and doxorubicin combined with CuE were used. It was found that, compared with the control group, all experimental groups significantly inhibited the growth of tumors in nude mice, and the combined drug group had the most obvious effect of inhibiting the growth of tumors in nude mice. The experimental groups inhibited the growth of tumor tissues of nude mice by exerting the chemotherapeutic effect of the drug; and the liver and kidney tissues of nude mice were not damaged while exerting the chemotherapeutic effect.

c-MYC is one of the members of MYC oncoprotein family that play critical role in various aspects of cancer biology including proliferation and chemoresistance.[19,20] Immunohistochemical detection of c-MYC expression in tumor tissues of nude mice revealed that CuE at a concentration of 75 μmol/L significantly reduced the expression of c-MYC protein in tumor tissues of nude mice compared with the control group, and Western Blot further detected the expression of ABCG2 and P-gp protein in tumor tissues of each group. In the experiments on nude mice, the combined drug group was more effective than the doxorubicin group alone in inhibiting tumor growth in nude mice, which could be explained by the fact that cucurbitacin could reduce the expression of c-MYC protein and drug-resistant protein ABCG2, P-gp protein in tumor tissues. The reason may be related to the fact that cucurbitacin can reduce the expression of c-MYC protein and drug resistance protein ABCG2 and P-gp in tumor tissues.

Based on the above experimental results, it was concluded that CuE could reduce the survival rate of laryngeal cancer stem cells, inhibit the proliferation ability of laryngeal cancer stem cells in vitro and inhibit the migration ability of laryngeal cancer stem cells, and CuE had a chemotherapeutic potentiation effect on doxorubicin, and the possible mechanism of the chemotherapeutic effect of CuE was to reduce the expression of c-MYC protein, and the possible mechanism of the chemotherapeutic potentiation effect was to reduce the expression of drug resistance protein ABCG2, P-gp protein expression.

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