Cancer Management and Research

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ORIGINAL RESEARCH

RETRACTED ARTICLE: Osthole sensitizes with radiotherapy to suppress tumorigenesis of human nasopharyngeal carcinoma in vitro and in vivo

This article was published in the following Dove Press journal:
Cancer Management and Research

Lin Peng[1,*], Yi-Teng Huang[2,*], Jian Chen[3], Yi-Xuan Zhuang[3], Fan Zhang[4], Jiong-Yu Chen[4], Li Zhou[5], Dong-Hong Zhang[6]

[1]Clinical Laboratory, Cancer Hospital of Shantou University Medical College, Shantou 515031, People's Republic of China; [2]Health Care Center, The First Affiliated Hospital of Shantou University Medical College. Shantou 515041, People's Republic of China; [3]Department of Radiotherapy, Cancer Hospital of Shantou University Medical College, Shantou 515031, People's Republic of China; [4]Oncological Research Lab, Cancer Hospital of Shantou University Medical College, Shantou 515031, People's Republic of China; [5]Department of Gynecological Oncology, Cancer Hospital of Shantou University Medical College, Shantou 515031, People's Republic of China; [6]Department of Cardiology, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou 325027, Zhejiang, People's Republic of China

*These authors contributed equally to this work

Correspondence: [illegible]
Oncological Research Lab, Cancer Hospital of Shantou University Medical College, No. 7 Raoping Road, Shantou 515031, People's Republic of China
Tel +86 754 8855 5844
Fax +86 754 8856 0352
Email kinyny@21cn.com

Dong-Hong Zhang
Department of Cardiology, The Second Affiliated Hospital of Wenzhou Medical University, 109 Xueyuan Road, Wenzhou 325027, Zhejiang, People's Republic of China
Tel/Fax +86 577 8800 2926
Email dzhang14@gsu.com

Background: Radiotherapy is one of the most common and useful treatments for nasopharyngeal carcinoma (NPC), but the radioresistance remains a major obstacle. Osthole, a natural coumarin derivative, has been shown to have anti-tumor and anti-inflammatory activity. However, the relationship between osthole and NPC treatment, especially for radiotherapy, is still elusive.

Methods: Osthole with or without X-ray radiotherapy treated with CNE2 cells, a human EC cell line. Cell viability, proliferation, migration and apoptosis were measured by MTT, colony formation, Annexin V/PI double staining, Transwell assay, respectively. NPC tumor models were established on BALB/c nude mice by subcutaneously injection of CNE2 cells and the effect of osthole and radiotherapy on tumor growth in vivo was studied.

Results: We found that in a dose-dependent manner, osthole could individually, and synergistically with radiotherapy, reduce NPC cell (CNE2) viability, proliferation, migration, and invasion, and induce apoptosis respectively. This effect of anti-tumor growth and induction of apoptosis was further confirmed in mice induced by subcutaneously injection with CNE2 cells and following treated with osthole or/and radiation.

Conclusion: Osthole increases the effect of radiotherapy on anti-human nasopharyngeal cancer.

Keywords: osthole, radiotherapy, human nasopharyngeal carcinoma, tumorigenesis, proliferation, apoptosis

Introduction

Human nasopharyngeal carcinoma (NPC) is the most frequent head and neck tumor in Southeast Asia, especially in South China.[1,2] Because of inherent anatomic location and radiosensitivity, radiotherapy with or without chemotherapy is the standard treatment for NPC.[3] However, radiation therapy is sometimes ineffective as cancer cells may be resistant to radiotherapy. Concurrent adjuvant chemotherapy was reported to improve the survival rate, and other treatment regimens are being persistently explored.[4] Thus, identification of effective and specific combined chemotherapy and radiotherapy regimens addresses an important unmet clinical need.

Osthole, extracted mainly from *Cnidium monnieri*, has been used as traditional Chinese medicine for the treatment of eczema, cutaneous pruritus, and *trichomonas vaginalis* infection. Previous studies have revealed that osthole exhibits various pharmacological activities, including anti-inflammation, anti-allergy, anti-oxidation, estrogen-like and anti-hepatitis effects.[5] Furthermore, accumulating evidence indicates that osthole confers anti-tumor and anti-metastatic activities by inducing apoptosis and cell cycle arrest in human lung cancer, breast carcinoma, head and neck squamous cell carcinoma, ovarian cancer, hepatocellular carcinoma, cervical

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http://dx.doi.org/10.2147/CMAR.S182798

cancer, colorectal adenocarcinoma, and glioblastoma multiforme.[6–13] In addition, osthole has recently been reported to induce neither apoptotic nor growth inhibiting effects on normal human peripheral blood mononuclear cells and cervical cells.[14]

However, the therapeutic efficacy of osthole against NPC and the possible mechanisms behind it remain unclear. Moreover, the efficacy of osthole combined with radiotherapy on NPC, to date, has not been examined. Here, we analyzed osthole and/or radiotherapy on human CNE2 NPC cell line in vitro and CNE2 tumors in vivo. Our study indicates that osthole not only inhibits human nasopharyngeal cancer but also increases the effect of radiotherapy.

Materials and methods

Cell culture

The human NPC cell line CNE2 was originally purchased from the Cell Bank, Chinese Academy of Sciences (Shanghai, People's Republic of China), and cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO_2. Cells were grown in sterile tissue culture dishes and passaged using 0.25% trypsin.

Cell viability assay

The CNE2 cell viability following treatment with osthole was measured by a MTT assay. Briefly, CNE2 cells were inoculated in 96-well plates at a density of 5,000 cells/well and allowed to attach overnight. The cells were treated with different concentrations of osthole (0, 10, 20, 40, 80, and 100 mM), with or without prior exposure to 2 or 5 Gy X-ray radiation. After incubation for 24, 48, and 72 hours, MTT (5 mg/mL) was added to each well and incubated for 4 hours, then cells were lysed by adding 100 µL of dimethyl sulfoxide. Absorbance was recorded on a microplate reader at a wavelength of 490 nm. Growth inhibition (%) was calculated as the fraction of (1-experimental OD/control OD) × 100%. A total of five replicates were performed for each.

Colony formation assay

CNE2 cells were inoculated at 200 cells/well into a six-well dish, treated with osthole or X-ray radiation, and incubated for 12 days to allow colony formation. Subsequently, the cells were fixed with methanol and stained with 0.1% crystal violet. Then, cells were manually counted under a dissecting microscope and clones were defined as groups of >50 cells. Each experiment was repeated three times.

Analysis of cell apoptosis

Briefly, CNE2 cells were inoculated into a six-well plate (50,000/well) and grown to ~70% confluence. Then, cells were treated with osthole and X-ray radiation individually or in combination for 24 hours, respectively. Apoptotic cells were quantified using an Annexin V/PI double-staining kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA), and samples were immediately analyzed by using a flow cytometer. Data were analyzed using FlowJo 7.6.2 software (FlowJo LLC, Ashland, OR, USA).

Western blot analysis

Osthole-treated and X-ray-irradiated cells were harvested in lysis buffer at 4°C for 1 hour. A BCA Protein Assay Kit was used for determining protein concentration (Beyotime Biotechnology, Jiangsu, People's Republic of China). A total of 20 µg protein was separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane. The membrane was incubated overnight at 4°C with antibodies against GAPDH, BCL-2, and BAX. Following incubation with peroxidase-conjugated anti-mouse/rabbit IgG (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at 37°C for 1 hour, proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL, USA) and detected using a bioimaging system (UVP Inc., Upland, CA, USA). The density of each band was quantified with ImageJ software and corrected by normalization to the expression value for GAPDH.[15,16]

Transwell assay

Cell invasion and migration of CNE2 cells were evaluated by a transwell assay (Corning, USA).[17] The upper chambers were coated with 40 mL Matrigel (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 1:3 dilution for the invasion assay or without Matrigel for the migration assay. For coating with Matrigel, the chambers containing Matrigel solution were incubated at 37°C for 2 hours. CNE2 cells were treated with osthole and X-ray irradiation alone or in combination for 24 hours, respectively. Cells remaining on the upper side of membrane were gently wiped, and cells that migrated to the lower side of the membranes were then fixed with 95% ethanol and stained with 1% crystal violet. Images of stained cells in five random fields were captured, using an optical microscope (Olympus Corporation, Tokyo, Japan), and counted.

In vivo tumor xenograft and treatment

Male BALB/c nude mice at 8 weeks of age were from Silaike Experimental Animal Company (Shanghai, People's Republic

of China). Individual mice were first injected subcutaneously with 2×10^6 CNE2 cells to establish xenograft tumors in vivo. Five days after injection, mice were randomized and treated with 0.9% NaCl (without radiation), osthole (1.5 mg/kg/2 days, intraperitoneal injection), or radiation (5 Gy/3 days for four times, total 20 Gy) alone, or a combination of osthole and radiation (n = 6 per group). The growth of implanted tumors was monitored every other day and the tumor volumes were calculated. The longest (length) and shortest (width) diameters of the tumor were assessed with digital calipers at regular intervals and their volumes were calculated according to the following formula: tumor volume = length × width$^2 \div 2$. Tumor growth curves were produced and data are presented as the mean ± SD. The animals were euthanized 4 weeks after the first inoculation and their tumors were frozen at −80°C.

All surgery procedures were performed in accordance with the guidelines and regulations of the Care and Use of Laboratory Animals by the US National Academy of Sciences and published by the US NIH (NIH publication 86-23 revised 1985). The study protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the ethics committee of Shantou University Medical College. Written confirmation of all experiments performed following guidelines and regulations of Shantou University Medical College was obtained prior to the commencement of this study.

Statistical analysis

Data were statistically analyzed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and expressed as the mean ± SD. All experiments were performed at least in triplicate, and differences between treatment groups were determined via one-way analysis of variance with post hoc contrasts by the Student–Newman–Keuls test. Statistical significance was accepted at $P < 0.05$.

Results

Osthole suppresses cell growth of human NPC in vitro

To determine the effects of osthole on human nasopharyngeal cancer, CNE2 cells were treated with osthole. MTT assay showed that osthole treatment inhibited the cell viability in a time- and dose-dependent manner (Figure 1A). Cell apoptosis was then determined by Annexin V/PI flow cytometry analysis, which indicated that CNE2 cells had increasing apoptosis with increasing concentrations of osthole (Figure 1B). Similarly, the suppression of CNE2 migration and invasion was also found by osthole treatment in a dose-dependent manner, as evidenced by a reduction in migrated and invaded cells to the bottom of the wells in the transwell assay as compared with the control (Figure 1C and D). These results show that osthole suppresses the tumorigenesis of human nasopharyngeal cancer in vitro.

Osthole increases the effect of radiotherapy on human NPC cells in vitro

Next, we evaluated whether osthole could increase the effect of radiotherapy on human nasopharyngeal cancer. CNE2 cells were treated with osthole and radiotherapy individually and in combination for 24 hours. As shown in Figure 2A–C, our MTT and cloning efficiency results showed that osthole (20 μg/mL) and radiotherapy (5 Gy but not 2 Gy) treatment alone inhibited cell viability and proliferation, respectively. Notably, combination treatment of osthole with radiotherapy inhibited cell proliferation to a greater extent than monotherapy. These results demonstrated that osthole and radiotherapy had a synergistic effect on the decrease in rate of CNE2 cell proliferation.

Moreover, a similar synergistic effect was also found for cell apoptosis, as determined by Annexin V/PI flow cytometry analysis. As shown in Figure 3A and B, the percentage of apoptotic cells induced by the combination of osthole (20 μg/mL) and radiotherapy (5 Gy) was 50.8% ± 4.2%, which was higher than that obtained from individual treatment with osthole (11.4% ± 2.0%) and radiotherapy (28.1% ± 1.7%). In addition, there was a significant increase of BAX (apoptosis marker) and decrease of BCL-2 (anti-apoptotic protein) following individual treatment with osthole (20 μg/mL) or radiotherapy (5 Gy). Much more significant changes were found by double treatment with osthole and X-rays (Figure 3C and D). These results demonstrated that osthole could increase the effect of radiotherapy treatment on human nasopharyngeal cancer in vitro.

Osthole and radiotherapy cooperatively suppress NPC growth in a murine tumor xenograft model of NPC

In order to explore the potential synergistic role of osthole and radiotherapy in nasopharyngeal cancer development in vivo, we next treated NPC tumors with osthole and radiotherapy in murine models. Nude mice were subcutaneously inoculated with 1×10^6 CNE2 cells (day 1). Tumors, from osthole-treated mice with or without X-ray treatment, were collected and weighed 21 days after tumor cell injection. As expected, there was a significant decrease of tumor growth by individual osthole or X-ray treatment. Similar to our in vitro results, the formation of tumors was further delayed by combined treatment with osthole and X-irradiation (Figure 4A and B). Furthermore, compared with the control group, osthole or

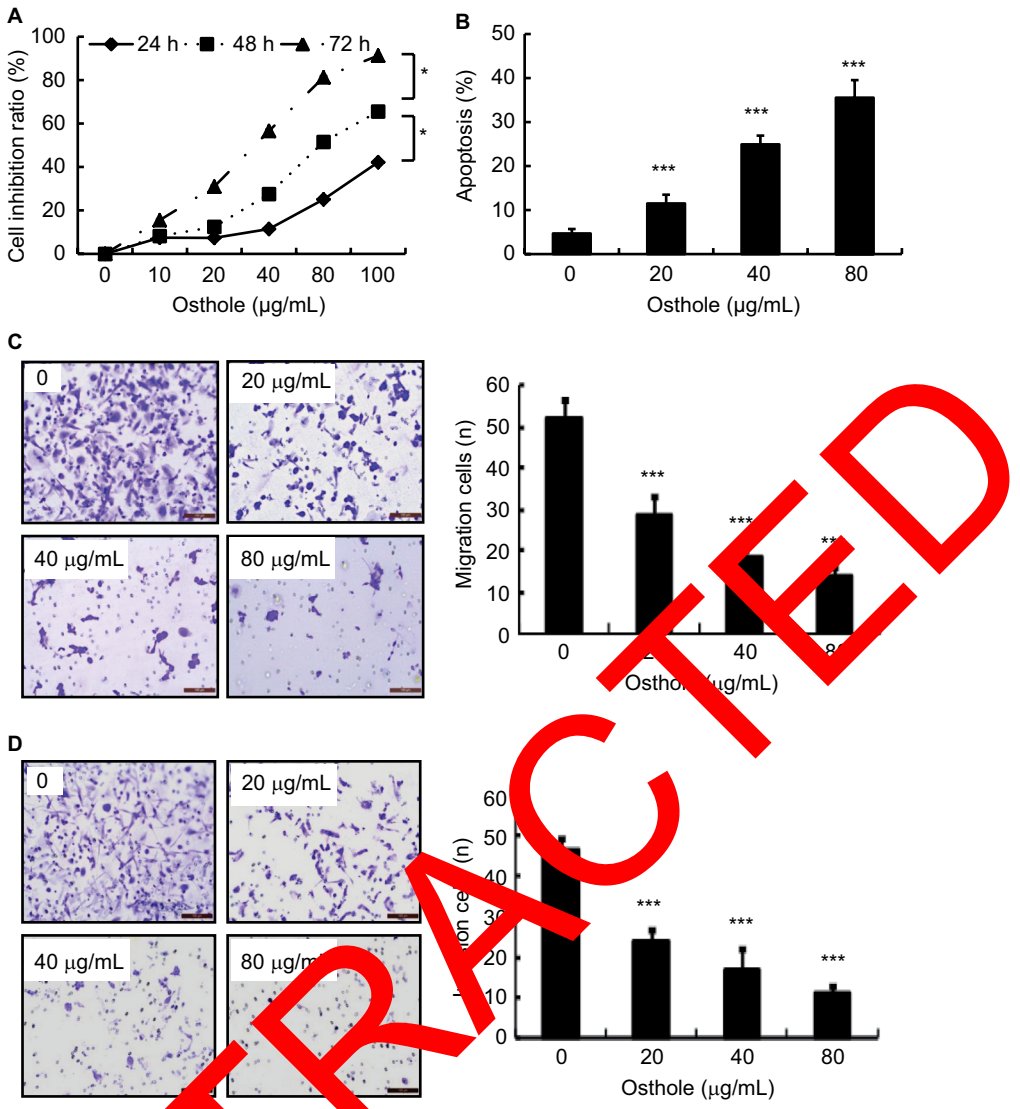

Figure 1 Osthole suppresses growth, migration, and invasion of human NPC in vitro.
Notes: (**A**) MTT assay was performed to measure cell growth at 24, 48, and 72 hours after osthole treatment. (**B**) Osthole-induced cell apoptosis was quantified using an Annexin V/PI double staining kit and analyzed by flow cytometry. Transwell assay with or without Matrigel for cell invasion (**C**) and migration (**D**). Representative transwell assay of cells following staining with crystal violet. The number of migrated cells was measured by counting five randomly chosen fields under a microscope. Bar = 50 μm. Data are mean ± SD from three independent experiments each performed in triplicate. ***$P<0.001$ compared with control.
Abbreviation: NPC, nasopharyngeal carcinoma.

X-ray treatment alone increased the expression of BAX, while BCL-2 levels were decreased following treatment. Notably, the osthole with radiotherapy combination treatment had a synergistic effect on the regulation of these proteins (Figure 4C and D). These results indicated that osthole and radiotherapy cooperatively suppressed nasopharyngeal tumor growth.

Discussion

We have tried to understand the molecular mechanism and cellular behavior during combination of radiotherapy with chemotherapy for the NPC, since the toxicity and adverse reactions are frequently unsatisfactory.[18] This present study is the first evidence of the anti-tumor effect of osthole, a natural coumarin derivative, and the cooperation with radiotherapy to suppress tumorigenesis, in human NPC. Our data suggest that osthole could be developed as a novel anti-tumor agent for treating NPC, especially combined with radiotherapy.

For the molecular mechanism and cellular behavior, our present study indicated that osthole and radiotherapy not only individually but also synergistically exhibit anticancer effects by inhibiting cell proliferation and migration and inducing cell apoptosis in NPC cell lines in vitro and in vivo. Our observation combined with previous reports indicated that osthole acts as a comment and widely tumor suppressor for various kinds of tumorigenesis.[6–13] Osthole has been found to exert health-promoting effects with a wide range of applica-

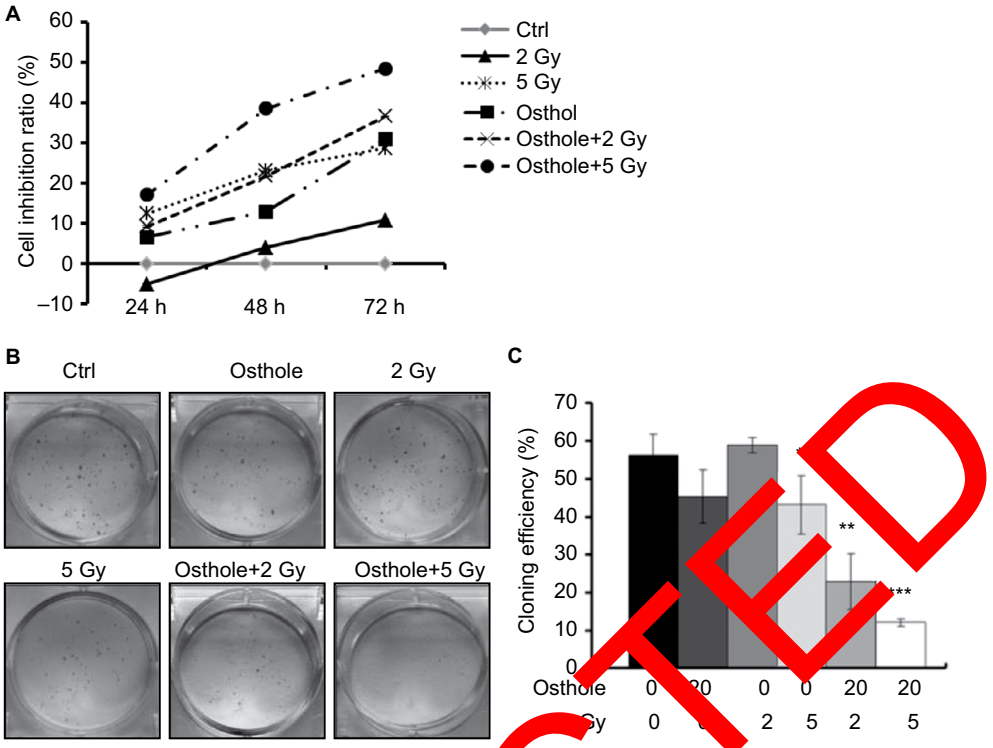

Figure 2 Osthole with radiotherapy has a synergistic effect on decreasing CNE2 cell proliferation.
Notes: MTT assay (**A**), colony formation assay (**B**), and its quantification (**C**) were performed to measure CNE2 cell growth following treatment with osthole or radiotherapy individually or combined. *P<0.05; **P<0.01; ***P<0.001 compared with control.
Abbreviation: Ctrl, control.

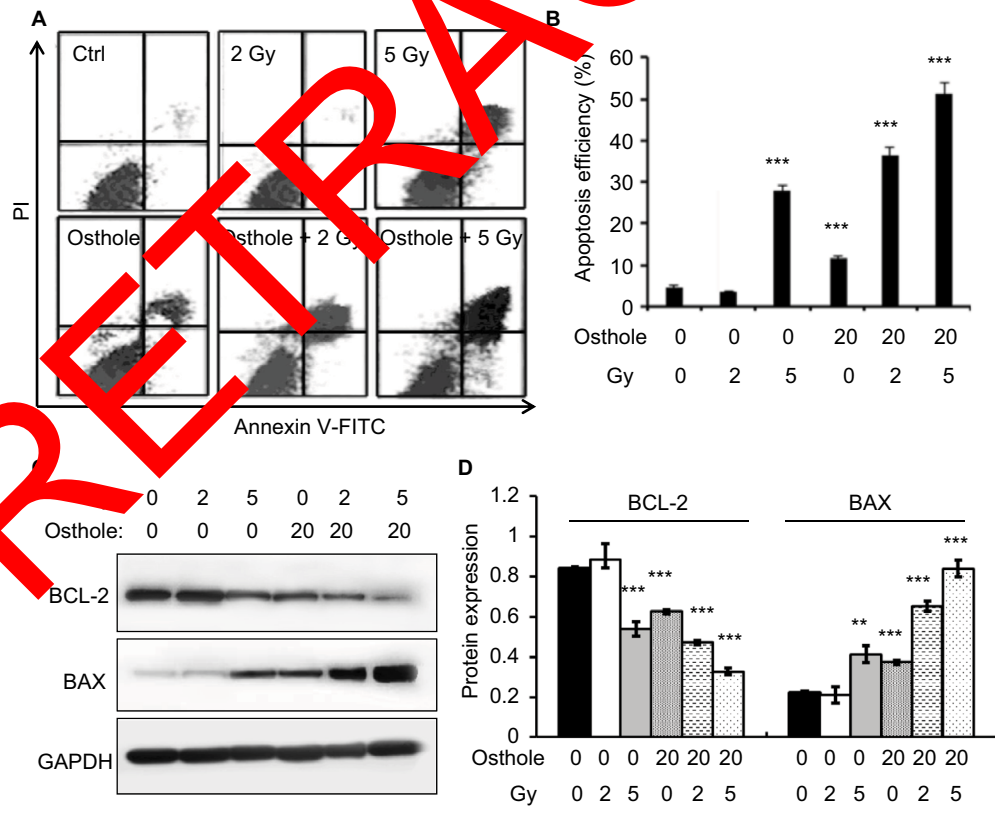

Figure 3 Combined effect of osthole and radiotherapy on CNE2 cell apoptosis.
Notes: Representative images (**A**) and quantification (**B**) of Annexin V/PI flow cytometry analysis of treatment with osthole and/or radiotherapy on CNE2 cells. Representative Western blot (**C**) and quantification (**D**) assay for pro-apoptotic of BAX and anti-apoptotic of BCL-2 expression. GAPDH was the normalized control. **P<0.01; ***P<0.001 compared with control.
Abbreviations: Ctrl, control; FITC, fluorescein isothiocyanate.

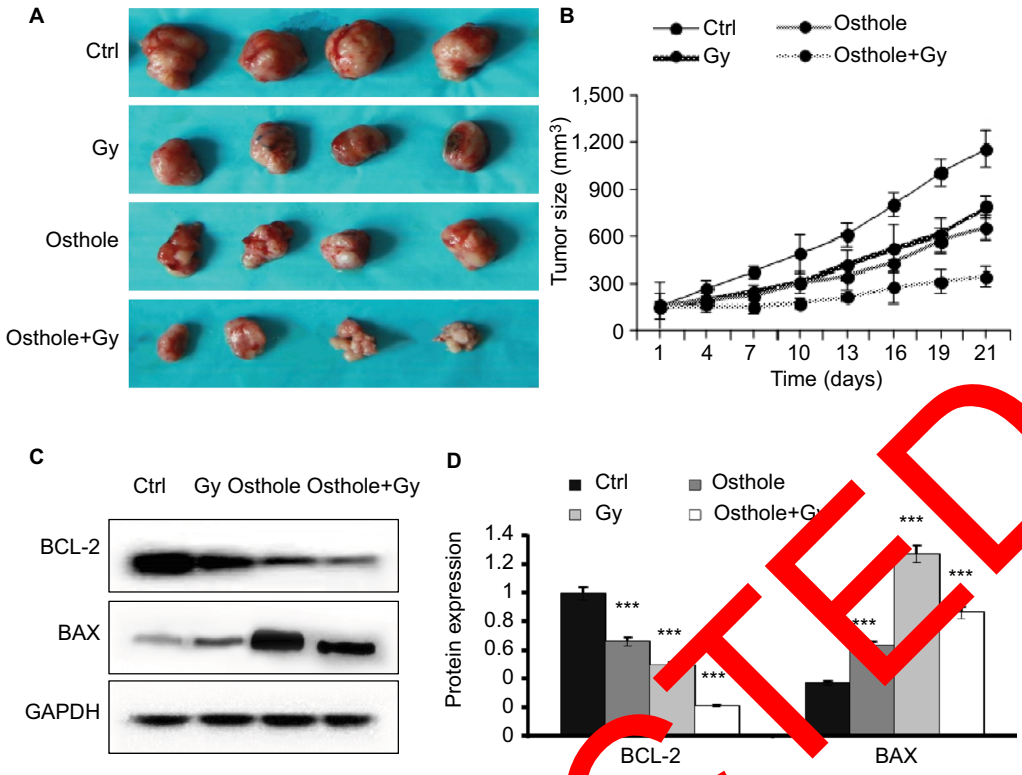

Figure 4 Osthole and radiotherapy cooperatively suppress NPC growth in vivo.
Notes: Representative images of tumor size (**A**) and tumor growth (**B**) following treatment with osthole and/or radiotherapy in a CNE2 tumor xenograft model. Representative Western blot (**C**) and quantification (**D**) assay for BAX and BCL-2 protein expression in tumor following osthole and/or radiotherapy treatment. GAPDH was the normalized control. ***$P<0.001$ compared with control.
Abbreviation: Ctrl, control.

tions, such as neuroprotective,[19] immunomodulatory,[20] and hepatoprotective.[21] The present data further show that osthole has strong anti-tumor effect by various behaviors including inhibition of cell viability, proliferation, migration, and invasion and induction of apoptosis.

Up to date, there is no report of osthole side effects on cancer treatment, so osthole is a safety and effective tumor suppressor. Moreover, osthole could successfully inhibit tumor synergistically with relatively low and safe Gy X-ray radiation for cellular or animal model, since we did not find any side effects for all mice treatment with X-ray radiations. This radio-sensitization effect is thought to be mediated by inhibition of S-phase cells with relative resistance to radiation. Although we did not detect changes in cell cycling following treatment with osthole on NPC cells, the anti-proliferative effect of osthole is indicative of induced cell cycle arrest. To date, apoptosis has been recognized as the most widely studied mechanism in anticancer therapy.[22] It is known that radiation induces DNA damage and leads to apoptosis. Reasonably, osthole individually or combined with radiation increases apoptosis of NPC cells via overexpression of BAX, accompanied by a reduction of BCL-2 in vivo and in vitro, which could explain the synergistic anticancer effect.[23] The side effects of radiation therapy in NPC, specifically the xerostomia, the severity late toxicities and poor quality of life has also greatly changed with the evolution of radiation treatment techniques. Indeed, some clinical trials using xerostomia, and its predictors, pretreatment factors, as well as different model of radiotherapy to reduce the side effects of radiation treatment.[24–27] Therefore, it is essential for oncologists to identify methods of improving the therapeutic efficacy and local control rate and to control the rate of distant metastasis and reduce the impairment of healthy tissues. Herein, our study supplies a novel anticancer agent, derived from herbal medicines, which could safely and successfully inhibit NPC tumorigenesis when combined with traditional radiotherapy.

Conclusion

Individually and combined with radiotherapy, osthole could effectively and safely fight against NPC and exert its effects by inhibiting cell proliferation and migration and inducing apoptosis in vitro and in vivo. The present study provides some cellular evidence underlying the radio-sensitizing effect of osthole, which could be used as a novel instruction for the further clinical trial of advanced NPC.

Acknowledgment

This work was supported by funds from The National Natural Science Foundation of China (No. 81602886), The National Natural Science Foundation of Guangdong province (No. 2016A030313062), and Science and Technology Planning Project of Shantou City, People's Republic of China (No. 201413).

Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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