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Effects of Cepharanthine Alone and in Combination with Fluoropyrimidine Anticancer Agent, S-1, on Tumor Growth of Human Oral Squamous Cell Carcinoma Xenografts in Nude Mice

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Abstract. *Background: Chemotherapy has shown little antitumor activity against advanced oral squamous cell carcinoma (OSCC) patients. Therefore, there is an urgent need to develop more effective therapeutic methods for patients with advanced OSCC. Cepharanthine is a biscoclaurine alkaloid extracted from Stephania cepharantha Hayata, which is widely used for the treatment of many acute and chronic diseases, and can exert antitumor effects on several human cancer cells. S-1 is a new oral antineoplastic agent that can induce apoptosis in various types of cancer cells, including OSCC. Hence combined treatment of cancer cells with cepharanthine and S-1 might exert dramatic antitumor effects on OSCC cells. Materials and Methods: In this study, the response of human OSCC cells to cepharanthine alone and in combination with S-1 was examined using nude mouse xenograft models. S-1 (10 mg/kg/day, 5 times/week) was administered orally and cepharanthine (20 mg/kg, 5 times/week) was injected into peritumoral tissue for three weeks. Apoptotic cells were detected by a TUNEL method. The protein expression of thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), and orotate phosphoribosyl transferase (OPRT) were assessed using immunohistochemistry; their gene expression was determined using microdissection and RT-PCR, and their protein levels using ELISA. Results: Combined therapy of cepharanthine and S-1 exerted antitumor effects on human OSCC xenografts markedly and significantly induced apoptotic cells in tumors treated with cepharanthine plus S-1. Immunohistochemistry showed that the expressions of TS and DPD were down-regulated, and that OPRT expression was up-regulated in tumors treated with cepharanthine plus S-1. In the same way, microdissection and RT-PCR revealed that the expression of TS and DPD mRNA was down-regulated and that expression of OPRT mRNA was up-regulated in tumors administered the combined treatment. Moreover, ELISA indicated that the protein levels of TS and DPD were down-regulated, and that OPRT was up-regulated in tumors treated with the combined therapy. During the experimental period, no loss of body weight was observed in mice treated with the combined therapy. Conclusion: These findings demonstrate that the combination of cepharanthine and S-1 is effective against OSCC and has the potential of being a new therapeutic tool for future treatment of these tumors.*

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Key Words: Cepharanthine, S-1, oral squamous cell carcinoma, apoptosis, TUNEL, combination therapy, xenografts, nude mice.

Carcinomas of the oral cavity accounted for 274,000 cases in 2002, with almost two-thirds of them in men. They are the 11th most common cancer in males and 13th in females in developed countries, and 6th in males and 10th in females in developing countries (1). Oral squamous cell carcinoma (OSCC) comprises the majority of oral carcinomas. OSCC is associated with a poor prognosis and high recurrence rate despite improvement in early diagnosis and multimodal treatments including surgery, chemotherapy and irradiation. In addition, extensive surgery or systemic chemotherapy is not often suitable for OSCC patients of advanced age or with complications. Therefore, in order to improve the prognosis of patients with OSCC, the development of new, effective chemotherapeutic agents has been a major goal.

S-1 is a novel orally administered anticancer drug that is a combination of tegafur (FU), 5-chloro-2, 4-

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dihydroxypyridine (CDHP) and oteracil potassium (Oxo) in a 1:0.4: 1 molar concentration ratio (2). CDHP is a competitive inhibitor of dihydropyrimidine dehydrogenase, which is involved in the degradation of 5-fluorouracil (5-FU), and acts to maintain efficacious concentrations of 5-FU in plasma and tumor tissues (3). Oxo, a competitive inhibitor of orotate phospho-ribosyltransferase, inhibits the phosphorylation of 5-FU in the gastrointestinal tract, reducing the serious gastrointestinal toxicity associated with 5-FU (4). S-1 therapy in athymic nude rats was associated with the retention of a higher and more prolonged concentration of 5-FU in plasma and tumor tissues when compared with tegafur/uracil (UFT) (5). The antitumor effect of S-1 has been already demonstrated in a variety of solid tumors: the response rates for advanced gastric cancer (6), colorectal cancer (7), non-small cell lung cancer (8), head and neck cancer (9) and oral cancer (10) in the phase II studies conducted in Japan were 44-49%, 35%, 22%, 29% and 41.5%, respectively. The efficacy of S-1 for the treatment of gastrointestinal cancer has also been reported in European patients: the response rates for advanced gastric cancer (11) and colorectal cancer (12) were 32% and 24%, respectively. Despite S-1 being effective against various cancers, systemic chemotherapy should be considered if S-1 single-agent therapy is inadequate against advanced or recurrent tumors.

Cepharanthine is one of the biscoclaurine alkaloids extracted from *Stephania cepharantha* Hayata (13), which has been widely used in Japan for treatment of many acute and chronic diseases; bronchial asthma, alopecia areata, leukopenia during radiation therapy or anticancer treatment (14). Recently, it has been reported that cepharanthine exerts antitumor effects by increasing the immunological competence of the host (15, 16). It has also been reported that cepharanthine increases the intracellular accumulation of the anticancer drug Adriamycin through inhibiting its efflux from tumor tissue (17), while reducing its intracellular accumulation in normal tissue (18). Cepharanthine might be able to circumvent doxorubicin-resistance (19, 20). Therefore, it is thought that cepharanthine may be useful in cancer chemotherapy. In addition, cepharanthine exerted antitumor effect by inducing apoptosis in a mouse leukemia cell line (21), an adenosquamous cell carcinoma cell line (22) and an oral squamous cell carcinoma cell line (23). However, little is known about the detailed mechanisms of antitumor activity of cepharanthine in solid tumors, including OSCC, and also about the combined effects of cepharanthine and S-1 against it.

Hence, in this study, whether the combination of cepharanthine and S-1 can augment S-1-induced apoptosis in OSCC xenografts was investigated. In addition, possible underlying mechanisms involved in the enhancement of apoptosis-inducing activity of S-1 were examined.

Materials and Methods

Cell lines and cell culture. B88 cells were isolated from an OSCC patient (24). HSC2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). B88 and HSC2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum 100 μg/ml streptomycin, 100 units/ml penicillin in a humidified atmosphere containing 5% CO_2.

Nude mice and tumor inoculations. Female athymic nude mice with CAnN.Cg-Foxnlnu/CrlCrlj genetic background (CLEA Japan, Inc. Tokyo, Japan) were purchased at 4 weeks of age and kept under sterile conditions in a pathogen-free environment. The mice were provided with sterile water and food *ad libitum* and all manipulations were carried out aseptically inside a laminar flow hood. Cells were used as a xenograft model in the nude mice. Briefly, tumor cells (1×10^6) were suspended in 0.1 ml of serum-free medium and injected into the subcutaneous tissue of 5-week-old nude mice (average weight 11.0 g) using a 27-gauge needle. Tumors were allowed to grow for 10 days before treatment. The mice were then divided into 4 groups, each of 5 mice with similar mean tumor volumes (100-150 mm^3).

Reagents and treatment protocol. Cepharanthine was obtained from Kaken Shoyaku Co. Ltd., (Tokyo, Japan). The reagent (20 mg/kg) was injected into peritumoral tissue for 3 weeks (5 times/week). S-1 was obtained from Taiho Pharmaceutical Co. Ltd., (Tokyo, Japan). The drug was suspended in autoclaved 0.5% sodium hydroxypropylmethylcellulose (HPMC; Daiichi seiyakukogyo, Kyoto, Japan) in sterile conditions, at 1.0 mg/mL, and subsequently homogenized by stirring. The suspension was given to mice *via* a gastric tube in a volume of 0.1 ml/10 g body weight for 3 weeks (5 times/week). Mice in the control group were orally administered an equal volume of 0.5% HPMC for 3 weeks (5 times/week), and received saline (200 μl) by peritumoral injection. Mice in the S-1 group were orally administered S-1 (10 mg/kg) for 3 weeks (5 times/week). Mice in the CE group received CE (20 mg/kg) for 3 weeks (5 times/week) by peritumoral injection. Mice in the S-1 plus CE group were orally administered S-1 (10 mg/kg) for 3 weeks (5 times/week), and received CE (20 mg/kg) for 3 weeks (5 times/week) by peritumoral injection. The tumors were measured every two days and the relative tumor volumes were calculated. At 21 days, mice were sacrificed by cervical dislocation and the tumors were dissected out, fixed in neutral-buffered formalin and embedded in paraffin for further study.

Microdissection. A representative formalin-fixed, paraffin-embedded (FFPE) tumor specimen, containing a central section of the cancer, was selected from each of the lesions by a pathologist after examination of the hematoxylin and eosin-stained slides. Sections of 10 μm were stained with nuclear fast red to enable visualization of histology for laser-captured microdissection (PALM Microlaser Technologies, Munich, Germany), which was performed to ensure both malignant cells and normal stromal cells were studied.

RNA extraction and cDNA synthesis from paraffin-embedded tissues. RNA was extracted according to the method of Chomczynski *et al*. with minor modifications (25). Briefly, 600 μl of xylene were added to each sample tube containing micro-dissected sections. After centrifugation for 7 min at 20,800×*g*, the supernatant was discarded,

and the washing step was repeated 3 times. The deparaffinized materials were rehydrated in xylene:ethanol:water at the following ratios (95:95:5, 95:90:10, 95:80:20, 95:75:25 and 95:70:30). After each step, the rehydration medium was removed after centrifugation for 7 min at 20,800×*g*. After discarding the last supernatant, the pelleted sections were redissolved in 70% ethanol. Subsequently, 400 µl of buffer (4 M guanidine isothiocyanate solution including 0.5% sarcosine and 8 µl of 1 M DTT) were added to the dried tissue which was homogenized mechanically. For RNA denaturation, homogenates were heated at 95˚C for 30 min. RNA was extracted from homogenates by the addition of 50 µl of 2 M sodium acetate (pH 4.0), 500 µl of water-saturated phenol and 100 µl of a chloroform-isoamyl mixture (49:1). RNA was recovered from the aqueous phase by isopropanol precipitation and transferred to a fresh tube and precipitated with 10 µl glycogen and 400 µl isopropanol for 30 min at –20˚C. After centrifuging for 7 min at 20,800×*g*, the pellet was washed with 500 µl 75% ethanol. After drying, the pellet was dissolved in 50 µl of 5 mM Tris HCl (pH 8.0). Reverse transcription was carried out at 39˚C for 45 min using 400 units of M-MLV reverse transcriptase 1×first-strand buffer, 0.04 µg/µl random hexamers, 10 mM DTT and 1 mM deoxynucleoside triphosphate (all reagents were purchased from Promega Corporation, Madison, WI, USA).

PCR quantification of mRNA expression. Target cDNA sequences were amplified by quantitative PCR using a fluorescence-based real-time detection method (ABI Prism 7900 Sequence Detection System, TaqMan; Applied Biosystems, Foster City, CA, USA). PCR was carried out for each gene of interest with *β-actin* as an internal reference gene. The 25 µl PCR reaction mixture contained 600 nmol/l of each primer, 200 nmol/l each of dATP, dCTP and dGTP, 400 mol/l dUTP, 5.5 mmol/l $MgCl_2$ and 1×TaqMan buffer A containing a reference dye (all reagents were purchased from Applied Biosystems). The primers and probe sequences used were as follows: TS primers: GCCTCGGTGTGCCTTTCA and CCCGTGATGTGCG CAAT, probe 6 FAM (carboxyfluorescein)-5-TCGCCAGCTACG CCCTGCTCA-3TAMRA (*N,N,N,N*-tetra methyl-6 carboxyrhodamine); DPD primers: AGGACGCAA GGAGGGTTTG and GTCCGCC GAGTCCTTACTGA, probe 6 FAM-5-CAGTGCCTACAGTCT CGAGTC TGCCAGTG3TAMRA; OPRT primers: TAGTGTTTTGG AAACTGTTGAGGTT and CTTGCCTCCCTGCTCTCTGT, probe 6FAM-TGGCATCAGTGA CCTTCAAGCCC TCCT; β-actin primers: TGAGCGCGG CTACAGCTT and TCCTTAATGTCACGC ACGATTT, probe 6FAM-5-ACCACCACGGCCGAGCGG-3TAMRA. The PCR conditions were 50˚C for 10 s and 95˚C for 10 min, followed by 42 cycles at 95˚C for 15 s and 60˚C for 1 min. The relative gene expressions of TS, DPD and OPRT were determined based on the threshold cycles of each gene in relation to the threshold cycle of the corresponding internal standard β-actin. The use of β-actin as a reference gene avoids the need for RNA concentration measurement. The β-actin real-time PCR analysis also estimated the amount of extracted mRNA. The rise of the β-actin signal after cycle 37 using the described conditions indicated an insufficient amount of mRNA present for the subsequent TS, DPD and OPRT quatification. When measuring gene expressions in paraffin-embedded tissues, the median value of the threshold cycle of β-actin was 26 cycles, ranging from 23 to 28, in malignant tissues.

Immunohistochemistry. The avidin-biotin complex immunohistochemical technique was used to detect TS, DPD and OPRT in tissue specimens, using the Vectastain kit (Vector Laboratories, Burlingame, CA, USA). Paraffin-embedded tissue sections 4 µm-thick were deparaffinized in xylene and rehydrated through graded alcohols. Endogenous peroxidase was quenched with a 0.3% hydrogen peroxide/methanol mixture for 30 min. Sections were rinsed and preincubated with 2% blocking serum for 30 min, followed by incubation with a TS, DPD and OPRT polyclonal antibody (obtained from Taiho Pharmaceutical Co. Ltd., Tokyo) for 8 h at 4˚C. After rinsing the tissue sections in phosphate-buffered saline (PBS) for 10 min, 100 µl of secondary antibody (biotinylated goat antirabbit) were added. After 30 min, tissue sections were again rinsed in PBS for 10 min and incubated with avidin-biotin complex for 30 min. Sections were rinsed in PBS and incubated with diaminobenzene substrate (Sigma, St. Louis, MI, USA) for 15 min. Tissues were finally rinsed in PBS for 5 min and tap water for 5 min, then counterstained with Mayer's hematoxylin (Sigma) for 1 min. The tissue sections were subsequently dehydrated in graded ethanol, cleared in Histoclear, and mounted with glass coverslips using DPX. Each run included positive and negative controls.

TUNEL (terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling) assay. To detect apoptotic cells, the ApopTag Plus peroxidase *in situ* Apoptosis Detection Kit (Intergen Company, Purchase, NY, USA) was used. Five-µm-thick paraffin sections of tumor were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Tissue sections were incubated in 20 µg/ml proteinase K (DAKO Corporation, Carpinteria, CA, USA) for 15 min. After sections were rinsed in distilled water, endogenous peroxidase was blocked by incubating the slides in 3% hydrogen peroxide in PBS (0.05 M phosphate buffer containing 0.145 M sodium chloride, pH 7.4) for 5 min. After being washed with PBS, the sections were incubated with equilibration buffer and then TdT enzyme in a humidified chamber at 37˚C for 60 min. They were subsequently put into prewarmed working strength stop wash buffer for 10 min. After being rinsed in PBS, the sections were incubated with antidigoxigenin-peroxidase conjugate for 30 min. Peroxidase activity in each section was demonstrated by the application of diaminobenzidine (Peroxidase Substrate Kit; Vector Laboratories). Hematoxylin was used as a counterstain. At least 1,000 cells were counted under a microscope in several random fields of each section. The number of apoptotic cells was divided by the total number of cells counted and the result was expressed as a percentage.

Enzyme-linked immunosorbent assay for TS, DPD and OPRT. The TS, DPD and OPRT expression levels in nude mouse tumors were each measured by enzyme-linked immunosorbent assay (ELISA) according to the methods of Mori *et al*. (26), Kurebayashi *et al*. (27) and Tsuji *et al*. (28), respectively.

Statistical analysis. All statistical significance was set at $p<0.05$. Statistical analyses were performed using StatView software (version 5.0J, SAS Institute Inc. Cary, NC, USA).

Results

Antitumor effects of cepharanthine alone and in combination with S-1 on tumor growth. Mice were treated with 20 mg/kg doses of cepharanthine alone and in combination with 10 mg/kg S-1 for 3 weeks (5 times/week) and tumor growth was observed during the treatment period

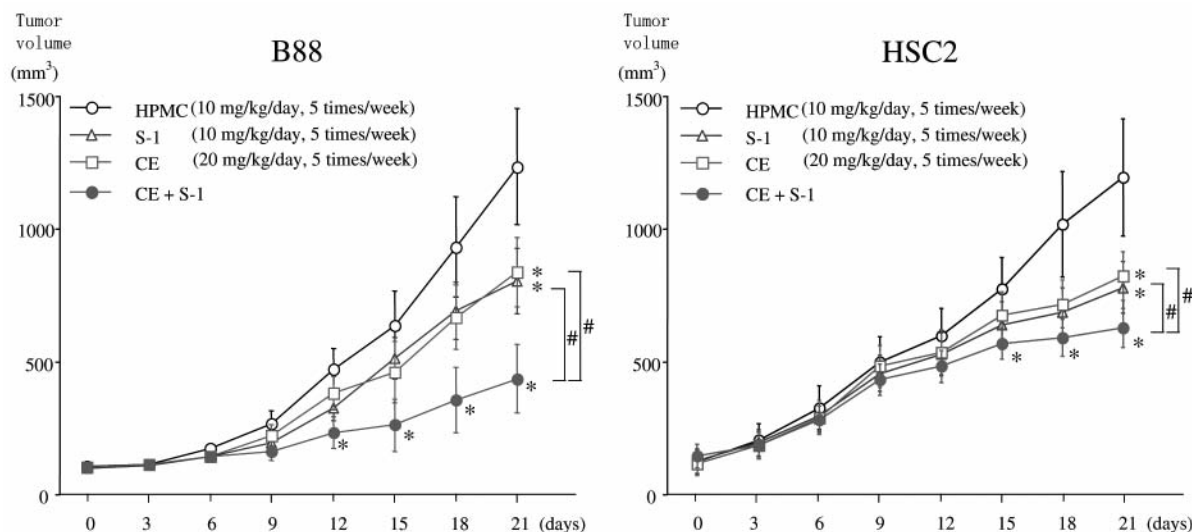


Figure 1. *Effect of cepharanthine (CE) alone, S-1 alone and combination with cepharanthine and S-1 on B88 and HSC2 tumor growth in nude mice. Growth inhibition effects of cepharanthine alone, S-1 alone, or cepharanthine plus S-1 were statistically significant when compared with that of the control (*p<0.01). The combination of cepharanthine and S-1 treatment was more effective than either agent alone (#p<0.01). Each group had 5 mice. The values shown are the mean of five tumors (mm³). Bars, SD.*

(Figure 1). Growth inhibition was seen with cepharanthine alone or S-1 alone in both tumor types, and this inhibition was statistically significant when compared with that of the controls. S-1 treatment was more effective than cepharanthine treatment. Moreover, the combination of cepharanthine and S-1 treatment was more effective than either agent alone. Furthermore, cepharanthine preferentially suppressed the growth of B88 tumors when compared with that of HSC2 tumors (Figure 1). No loss of body weight was observed in mice treated with cepharanthine and/or S-1 during the experimental period in either tumor types (Figure 2). Therefore, the B88 tumors were used for further investigation.

Analysis of apoptosis after cepharanthine alone and in combination with S-1 on nude mice tumor. To analyze the degree of apoptosis, tumors were removed from mice after treatment and the number of apoptotic cells was quantified by the TUNEL assay. The degree of apoptosis in the tumors treated with either cepharanthine or S-1 alone was significantly higher than that seen in the control group. The largest number of apoptotic cells was seen in the tumors treated with the cepharanthine and S-1 combination. This combination treatment significantly induced apoptosis compared to each agent alone as well as to the control (Figure 3). The extent of apoptosis (number of TUNEL-positive cells) in the groups with B88 tumors was as follows: Control group, 0.7±0.67; S-1 group, 11.4±1.71; Cepharanthine group 12.5±1.58; Cepharanthine plus S-1, 17.8±2.04 (Figure 3).

Expression of TS, DPD and OPRT in nude mice tumors. B88 tumors were examined for expression of TS, DPD and OPRT by immunohistochemistry. In immunohistochemical staining, TS expression was reduced markedly in cepharanthine-treated tumors and cepharanthine plus S-1-treated tumors (Figure 4). DPD expression decreased in cepharanthine, S-1 and cepharanthine plus S-1-treated tumors (Figure 4). OPRT expression was increased slightly in cepharanthine, S-1 and cepharanthine plus S-1-treated tumors (Figure 4). Briefly, combined treatment with cepharanthine and S-1 induced down-regulation of TS and DPD expression and up-regulated OPRT expression in B88 tumors.

Gene expression levels of TS, DPD and OPRT in nude mouse tumor. B88 tumors were also examined for gene expression levels of TS, DPD and OPRT by microdissection and real-time RT-PCR. *TS* mRNA expression was reduced in S-1-treated tumors and even more reduced in cepharanthine or cepharanthine plus S-1-treated tumors. *DPD* mRNA expression was reduced in cepharanthine, S-1 and cepharanthine plus S-1-treated tumors. *OPRT* mRNA expression was increased slightly in S-1, cepharanthine and cepharanthine plus S-1- treated tumors. In short, combined treatment of cepharanthine and S-1 also induced down-regulation of *TS* mRNA and *DPD* mRNA expression and the up-regulation of *OPRT* mRNA expression in B88 tumors similarly to each protein expression level (Table I).

Protein levels of TS, DPD and OPRT in nude mouse tumor. The protein levels of TS, DPD and OPRT in B88 tumors were measured by ELISA. The TS protein level was lower

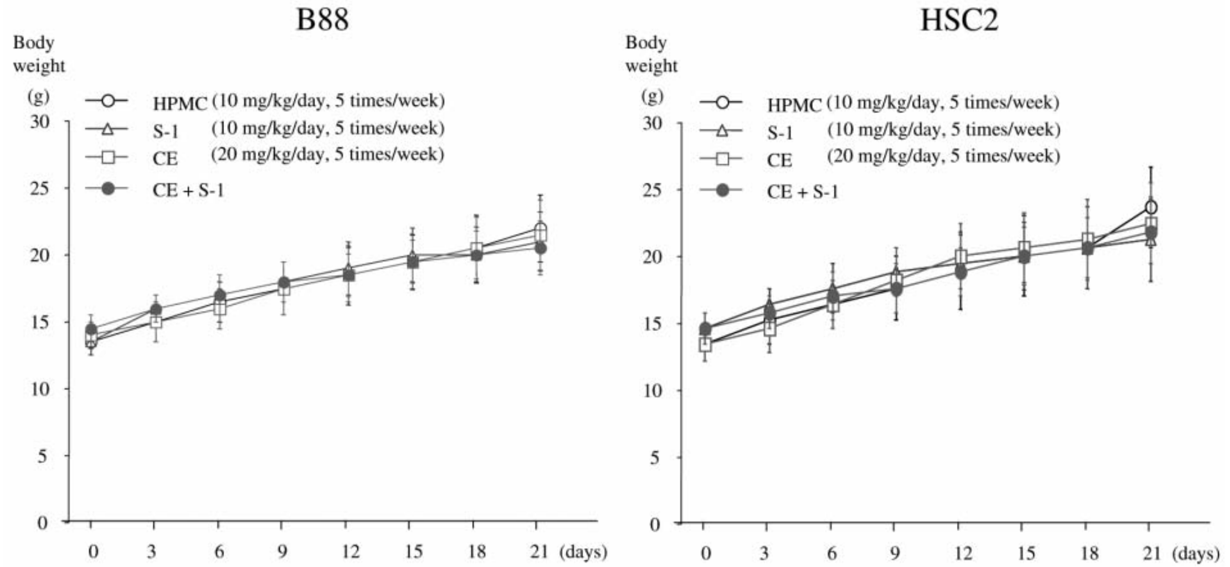
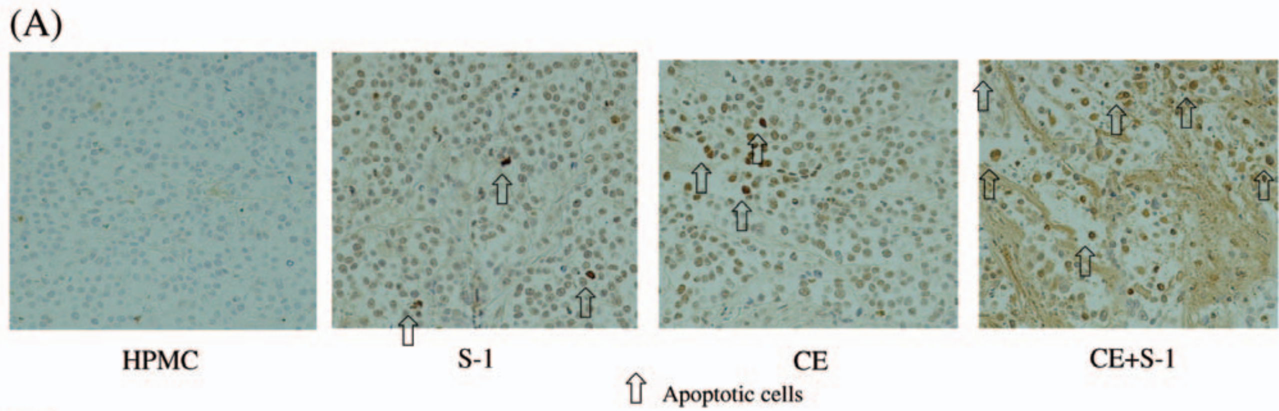


Figure 2. *Body weight of nude mice bearing B88 or HSC2 tumors. During the experimental period no loss of body weight was observed in mice treated with cepharanthine (CE) and/or S-1. Bars, SD.*



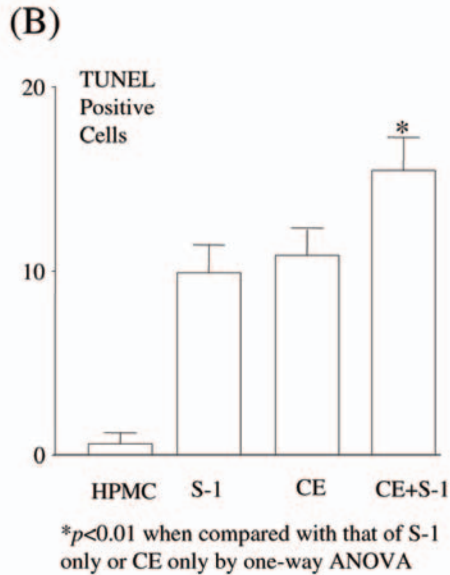


Figure 3. *(A) TUNEL assay. The largest number of apoptotic cells was seen in the tumors treated with the cepharanthine and S-1 combination (CE+S-1). Arrows show apoptotic cells (original magnification ×300). (B) Analysis of the degree of apoptosis present in tumors with cepharanthine alone, S-1 alone and cepharanthine plus S-1. The cepharanthine and S-1 treatment more markedly induced apoptosis than either agent alone (*$p<0.01$). Bars, SD.*

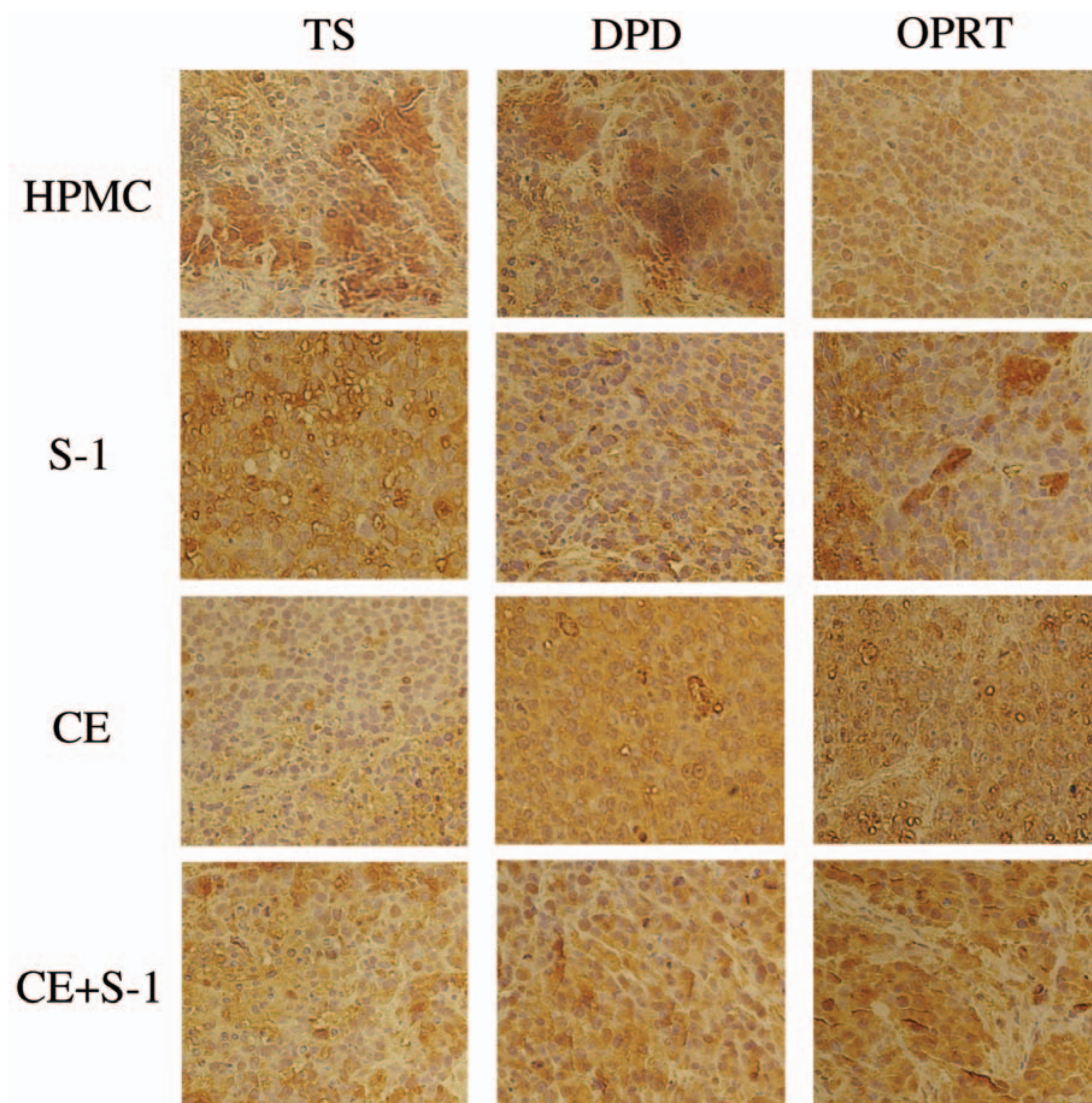


Figure 4. *Expression of TS, DPD and OPRT in nude mouse tumors treated with cepharanthine alone, S-1 alone and cepharanthine plus S-1. TS expression was reduced markedly in cepharanthine- and cepharanthine plus S-1-treated tumors. DPD expression was reduced in cepharanthine, S-1 and cepharanthine plus S-1-treated tumors. OPRT expression was increased slightly in cepharanthine, S-1 and cepharanthine plus S-1-treated tumors.*

in S-1-treated tumors and even more reduced in cepharanthine- or cepharanthine plus S-1-treated tumors. The DPD protein level was lower in cepharanthine-, S-1- and cepharanthine plus S-1-treated tumors. The OPRT protein level was slightly higher in S-1-, cepharanthine- and cepharanthine plus S-1-treated tumors. Briefly, the combined treatment of cepharanthine and S-1 induced the down-regulation of TS protein and DPD protein and the up-regulation of OPRT protein level in B88 tumors similarly to each protein expression (Table II).

Discussion

Although the currently used combined modality therapies have improved the outcome of patients with advanced OSCC, their efficacies are still limited and may cause serious side-effects or oral dysfunctions. Therefore, there is general agreement for the need of identification of new therapeutic targets and development of new agents with high efficacy and tolerability. Recently, a wide variety of factors involved in the prediction of chemotherapy with 5-FU against

Table I. *Gene expression levels of TS, DPD and OPRT relative to β-actin in nude mouse tumors with the treatments shown.*

Treatment	TS	DPD	OPRT
HPMC	25.78	3.21	2.69
S-1 (10 mg/kg)	19.99	2.42	2.78
CE (20 mg/kg)	10.40	2.73	3.11
CE+S-1	14.36	2.45	3.04

Table II. *Protein levels (ng/mg protein) of TS, DPD and OPRT in nude mouse tumors with the treatments shown.*

Treatment	TS	DPD	OPRT
HPMC	733.0	55.3	14.5
S-1 (10 mg/kg)	585.1	44.4	14.8
CE (20 mg/kg)	421.9	48.4	15.7
CE + S-1	532.1	46.6	14.9

gastrointestinal carcinoma including OSCC were identified (29). The degree of TS, DPD or OPRT protein expression is thought to be one of the most reliable factors of all. Many studies have reported that various carcinomas with higher expression of TS, DPD and lower expression of OPRT may resist 5-FU-based chemotherapy (30-32). These findings have led to the investigation of new therapeutic agents which can down-regulate TS and DPD expression and up-regulate OPRT expression against OSCC cells.

In the present study, we examined the response of human OSCC cells to cepharanthine alone and in combination with S-1 using nude mice xenograft models. An investigation was also performed on the mechanisms of the antitumor effects of cepharanthine in combination with S-1. The results demonstrated that cepharanthine alone significantly inhibited the growth of B88 and HSC2 tumors when compared with that of the controls. The antitumor activity of cepharanthine was greatly augmented by its use in combination with S-1. The mechanism of augmentation of the antitumor activity by combined therapy was related to induction of apoptosis (Figure 3). Interestingly, combined therapy of cepharanthine and S-1 as well as cepharanthine alone were able to induce the down-regulation of TS and DPD expression and the up-regulation of OPRT expression both at the protein and mRNA level. Cepharanthine alone induced down-regulation of TS expression and the up-regulation of OPRT expression to a greater extent than combined therapy with cepharanthine and S-1 (Figure 4; Tables I and II). These findings suggest that combined therapy with cepharanthine and S-1, as well as with cepharanthine alone may lead to the enhancement of chemosensitivity to S-1 in xenograft tumors and further, that pretreatment with cepharanthine may be more effective than concurrent treatment. Nevertheless, combined therapy with cepharanthine and S-1 may exert beneficial antitumor effects on patients with advanced OSCC resistant to S-1. In this respect, combined therapy with cepharanthine and S-1 may have wide clinical applications.

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