

## Effects of Tumor Necrosis Factor-related Apoptosis-inducing Ligand Alone and in Combination with Fluoropyrimidine Anticancer Agent, S-1, on Tumor Growth of Human Oral Squamous Cell Carcinoma Xenografts in Nude Mice

YASUTAKA ITASHIKI, KOJI HARADA, TARANNUM FERDOUS and HIDEO YOSHIDA

*Department of Therapeutic Regulation for Oral Tumors, Institute of Health Bioscience, University of Tokushima Graduate School, Tokushima, Japan*

**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. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor ligand family that selectively induces apoptosis of 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 TRAIL and S-1 might exert dramatic antitumor effects on OSCC cells. *Materials and Methods:* In this study, the response of human OSCC cells to TRAIL 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 TRAIL (1 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 TRAIL and S-1 exerted antitumor effects on human OSCC xenografts markedly and significantly induced apoptotic cells in tumors treated with TRAIL 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 TRAIL 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 TRAIL and S-1 is effective against OSCC and has the potential of being a new therapeutic tool for future treatment of these tumors.

Oral squamous cell cancer (OSCC) is associated with a poor prognosis and high recurrence rate despite improvement in early diagnosis and multimodal treatments including operation, chemotherapy and irradiation. In addition, extensive operation or systemic chemotherapy is not often suitable for OSCC patients of advanced age or with complications. 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 (FT), 5-chloro-2, 4-dihydropyridine (CDHP) and oteracil potassium (Oxo) in a 1:0.4:1 molar concentration ratio (1). 5-Chloro-2, 4-dihydropyridine 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 (2). Oteracil potassium, a competitive inhibitor of orotate phosphoribosyltransferase, inhibits the phosphorylation of 5-FU in the gastrointestinal tract, reducing the serious gastrointestinal toxicity associated with 5-FU (3). 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

*Correspondence to:* Dr. Koji Harada, Department of Therapeutic Regulation for Oral Tumors, Institute of Health Bioscience, University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan. Tel: +81 88 633 7354, Fax: +81 88 633 7462, e-mail: harako@dent.tokushima-u.ac.jp

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

compared with tegafur/uracil (UFT) (4). The antitumor effect of S-1 has been already demonstrated in a variety of solid tumors: the response rates for advanced gastric cancer (5), colorectal cancer (6), non-small cell lung cancer (7) and head and neck cancer (8) in the late phase II studies conducted in Japan were 44-49%, 35%, 22%, and 29%, 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 (9) and colorectal cancer (10) were 32% and 24%, respectively. S-1 is effective against various cancers as described above, however, systemic chemotherapy should be considered when S-1 single-agent therapy is inadequate against advanced or recurrent tumors.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a member of the TNF family and has been shown to selectively induce apoptosis in cancer cells with minimal or no toxicity against normal tissues, as examined both *in vitro* and *in vivo* in mice (11, 12). Therefore, TRAIL has been a promising target for molecular targeting therapy against various carcinomas. Moreover, the combination of TRAIL and a chemotherapeutic agent, such as 5-FU, *cis*-diamminedichloroplatinum(II), doxorubicin and CPT-11 was shown to augment TRAIL-induced apoptosis in some human cancer cells (13-17). This synergy was also observed in multidrug-resistant cell lines (18) or TRAIL-resistant cell lines (13, 18, 19). Interestingly, Mizutani *et al.*, reported that a combination of TRAIL and 5-FU was able to overcome the resistance of renal cell carcinoma to TRAIL or 5-FU by down-regulating the expression of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) modestly, and up-regulating the expression of orotate phosphoribosyltransferase (OPRT) (20). Briefly, they showed that TRAIL could enhance the sensitivity of renal cell carcinoma to 5-FU by regulating the predictive factors of 5-FU. S-1 has cytotoxic mechanisms similar to those of 5-FU though S-1 has shown antitumor activity far superior to that observed for 5-FU against human tumor xenografts (21, 22). However, little is known about the combined effects of TRAIL and S-1 against OSCC. TRAIL may enhance the sensitivity of OSCC to S-1 by regulating the predictive factors of 5-FU.

Hence, in this study, whether the combination of TRAIL 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 oral SCC patient in our laboratory (23). 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<sub>2</sub>/95% air.

**Nude mice and tumor inoculations.** Female athymic nude mice with CAnN.Cg-Foxn1nu/Cr1Cr1j 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 (1x10<sup>6</sup>) were suspended in 0.1 ml of serum-free medium and injected into the subcutaneous tissue of 5-week-old nude mice (average weight 10.4 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<sup>3</sup>).

**Reagents and treatment protocol.** TRAIL (1 µg/kg – 10 mg/kg) (Calbiochem, San Diego, CA, USA) 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). Control mice were administered orally with an equal volume of 0.5% HPMC for 3 weeks (5 times/week). Mice in control groups received saline (200 µl) 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 malignant cells and normal stromal cells were studied.

**RNA extraction and cDNA synthesis from paraffin-embedded tissues.** A representative formalin-fixed, paraffin-embedded tumor specimen, which contained 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. Ten µm thick sections 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 malignant cells and normal stromal cells were studied. RNA was extracted according to Chomczynski *et al.*'s method with minor modifications (24). Briefly, 600 µl of xylene was added to each sample tube containing micro-dissected sections. After centrifugation for 7 min at 20,800 xg, 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 xg. After discarding the last supernatant, the pelleted sections were redissolved in 70% ethanol. Subsequently, 400  $\mu$ l of buffer (4 M guanidine isothiocyanate solution including 0.5% sarcosine and 8  $\mu$ l 1 M DTT) were added to the dried tissue and homogenized mechanically. For RNA denaturation, homogenates were heated at 95°C for 30 min. RNA was extracted from homogenates by the addition of 50  $\mu$ l of 2 M sodium acetate (pH 4.0), 500  $\mu$ l of water-saturated phenol and 100  $\mu$ l of a chloroform-isoamyl mixture (49:1). RNA was recovered from the aqueous phase by isopropanol precipitation and transferred to a fresh tube and precipitate with 10  $\mu$ l glycogen and 400  $\mu$ l isopropanol for 30 min at -20°C. After centrifuging for 7 min at 20,800 xg, the pellet was washed with 500  $\mu$ l 75% ethanol. After drying, the pellet was dissolved in 50  $\mu$ l 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 x first-strand buffer, 0.04  $\mu$ g/ $\mu$ l 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  $\beta$ -actin was used as an internal reference gene. The 25  $\mu$ l 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<sub>2</sub> and 1 x TaqMan buffer A containing a reference dye (all reagents were purchased from Applied Biosystems). The primers and probe sequences used were follows: TS primers: GCCTCGGTGTGCCTTTCA and CCCGTGATGTGCGC AAT, probe 6 FAM (carboxyfluorescein)-5-TCGCCAGCTACG CCCTGCTCA-3TAMRA (*N,N,N,N*-tetra methyl-6 carboxyrhodamine); DPD primers: AGGACGCAA GGAGGG TTG and GTCCGCCGAGTCCTTACTGA, probe 6 FAM-5-CAGTGCC TACAGTCT CGAGTC TGCCAGTG3TAMRA; OPRT primers: TAGTGTTTTGGAAACTGTTGAGGTT and CTTGCCTCCCT GCTCTCTGT, probe 6FAM-TGGCATCAGTGACCTTCAAGC CC TCCT;  $\beta$ -actin primers: TGAGCGCG GCTACAGCTT and TCCTTAATGTCACGC ACGATTT, probe 6FAM-5-ACCACCA CGGCCGAGCGG-3TAMRA. The PCR conditions were 50°C for 10 sec and 95°C for 10 min, followed by 42 cycles at 95°C for 15 sec 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  $\beta$ -actin. The use of  $\beta$ -actin as a reference gene avoids the need for RNA concentration measurement. The  $\beta$ -actin real-time PCR analysis also estimated the amount of extracted mRNA. The rise of the  $\beta$ -actin signal after cycle 37 using the described conditions indicated an insufficient amount of mRNA present for the subsequent TS, DPD and OPRT quantification. When measuring gene expressions in paraffin-embedded tissues, the median value of the threshold cycle of  $\beta$ -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  $\mu$ m-

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  $\mu$ l of secondary antibody (biotinylated goat antirabbit) was added for 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- $\mu$ m-thick paraffin sections of tumor were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Tissue sections were incubated in 20  $\mu$ g/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 1000 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 mice tumors were measured by enzyme-linked immunosorbent assay (ELISA) according to the method of Mori *et al.*, (25), Kurebayashi *et al.*, (26) and Tsuji *et al.* (27). TS expression level was measured by the method of Mori *et al.*, and DPD expression level was measured by the method of Kurebayashi *et al.* and OPRT expression level was measured by the method of Tsuji *et al.*

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

## Results

**Effects of TRAIL alone on tumor growth and body weight.** B88 tumors were treated with various concentrations of TRAIL (1  $\mu$ g/kg to 10 mg/kg) for 3 weeks (5 times/week) and growth inhibitory effects were observed in 1 to 10 mg/kg TRAIL-

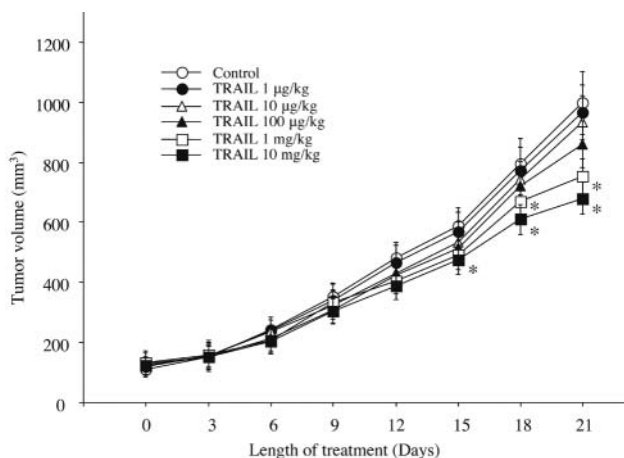


Figure 1. Effect of TRAIL alone on B88 tumor growth in nude mice. B88 tumors were treated with various concentrations of TRAIL. Growth inhibition effects of TRAIL alone (1-10 mg/kg) were statistically significant when compared with that of control (\* $p < 0.01$ ). Each group had 5 mice. The values shown are the mean of five tumors ( $\text{mm}^3$ ). Bars, SD.

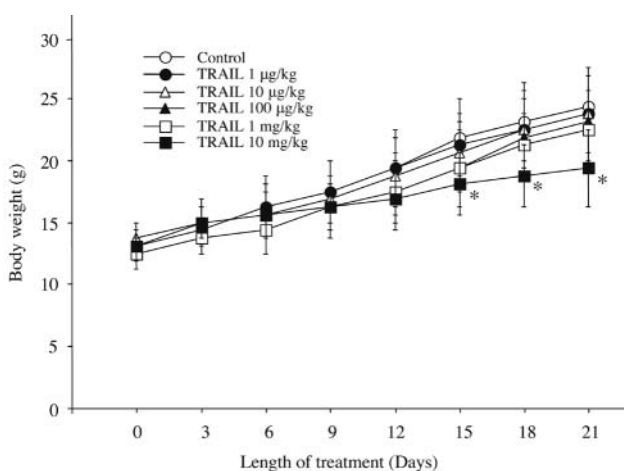


Figure 2. Body weight of nude mice treated with various concentrations of TRAIL. The body weight of mice treated with TRAIL (10 mg/kg) was significantly lower when compared with that of control (\* $p < 0.01$ ). Bars, SD.

treated nude mice (Figure 1). However, the body weight of mice treated with TRAIL (10 mg/kg) was significantly reduced when compared with that of controls (Figure 2). Therefore, we decided to use the concentration of 1 mg/kg TRAIL, which is the maximum concentration that did not show a loss of body weight of nude mice, for further investigation.

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

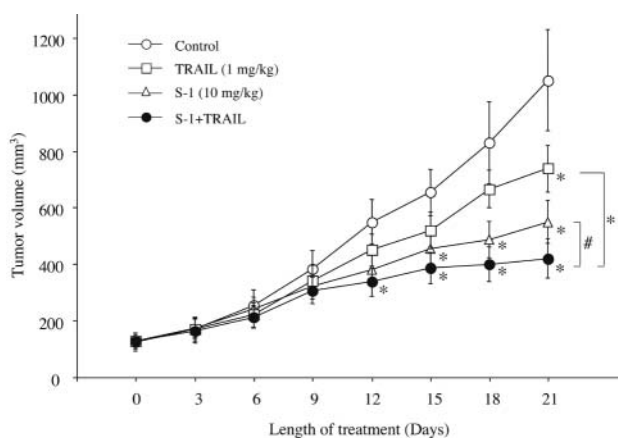


Figure 3. Effect of TRAIL alone and in combination with S-1 on B88 tumor growth in nude mice. Growth inhibition effects of TRAIL alone, S-1 alone, or TRAIL plus S-1 were statistically significant when compared with that of control (\* $p < 0.01$ ). The combination of TRAIL and S-1 treatment was more effective than either agent alone (\* $p < 0.01$ , #;  $p < 0.05$ ). Bars, SD.

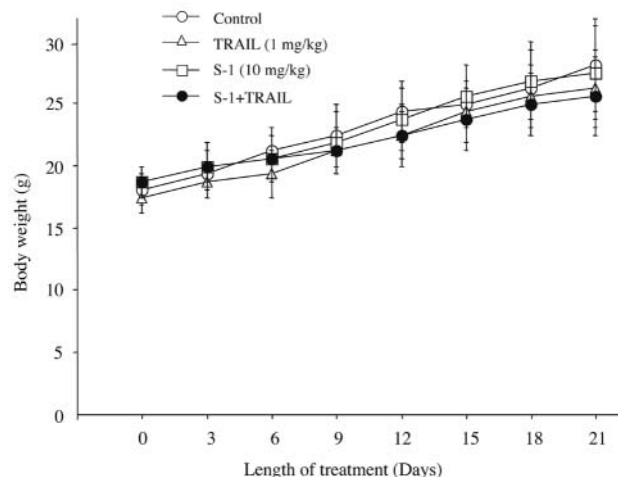


Figure 4. Body weight of nude mice bearing B88 tumors. During the experimental period, no loss of body weight was observed in mice treated with TRAIL and/or S-1. Bars, SD.

during the treatment period (Figure 3). Growth inhibition was seen with TRAIL alone or S-1 alone and this inhibition was statistically significant when compared with that of controls. Moreover, S-1 treatment was more effective than TRAIL treatment. Moreover, the combination of TRAIL and S-1 treatment was more effective than either agent alone. Furthermore, TRAIL preferentially suppressed the growth of B88 tumors when compared with that of HSC2 tumors (Figure 5). No loss of body weight was observed in mice treated with TRAIL and/or S-1 during the experimental period (Figures 4, 6).

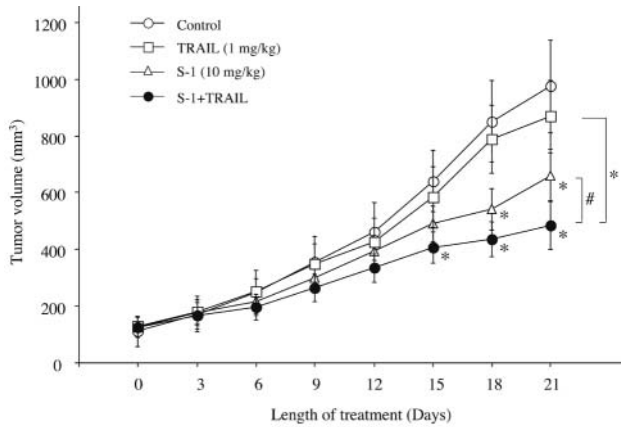


Figure 5. Effect of TRAIL alone and in combination with S-1 on HSC2 tumor growth in nude mice. Growth inhibition effects of TRAIL alone, S-1 alone, or TRAIL plus S-1 were statistically significant when compared with that of control (\* $p < 0.01$ ). The combination of TRAIL and S-1 treatment was more effective than either agent alone (\* $p < 0.01$ ; # $p < 0.05$ ). Bars, SD.

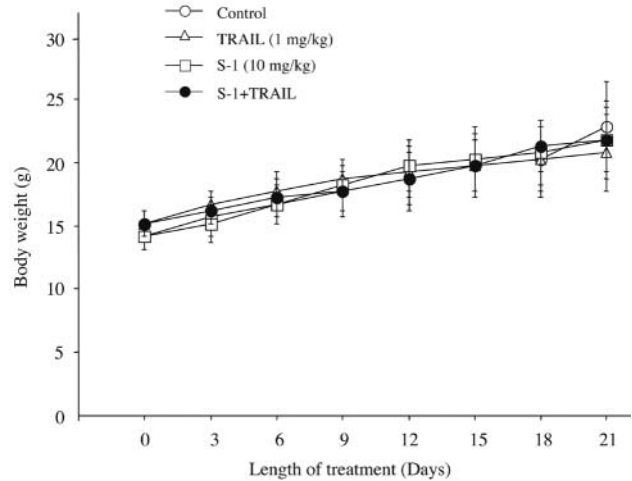


Figure 6. Body weight of nude mice bearing HSC2 tumors. During the experimental period, no loss of body weight was observed in mice treated with TRAIL and/or S-1. Bars, SD.

*Analysis of apoptosis after TRAIL 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 TRAIL 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 TRAIL and S-1 combination. This combination treatment significantly induced apoptosis compared to each agent alone as well as the control (Figure 7). The extent of apoptosis (number of TUNEL positive cells) in these groups of B88 tumors was as follows: Control group,  $1.18 \pm 0.5$ ; S-1 group,  $7.77 \pm 2.22$ ; TRAIL group  $8.53 \pm 1.73$ ; TRAIL plus S-1,  $18.2 \pm 2.01$  (Figure 8).

*Expression of TS, DPD and OPRT in nude mice tumors.* We examined B88 tumors for expression of TS, DPD and OPRT by immunohistochemistry. In immunohistochemical staining, TS expression was reduced slightly in TRAIL-treated tumors, and markedly in TRAIL plus S-1-treated tumors (Figure 9). DPD expression was decreased in TRAIL, S-1 and TRAIL plus S-1-treated tumors (Figure 10). OPRT expression was increased slightly in TRAIL treated tumors and S-1-treated tumors, and markedly in TRAIL plus S-1-treated tumors (Figure 11). Briefly, combined treatment of TRAIL and S-1 induced down-regulated TS and DPD expression and up-regulated OPRT expression in B88 tumors.

*Gene expression levels of TS, DPD and OPRT in nude mice tumor.* We also examined B88 tumors for gene expression levels of TS, DPD and OPRT by microdissection and

Table I. Gene expression levels of TS, DPD and OPRT in nude mouse tumors treated with TRAIL or S-1, alone and in combination.

Treatment	TS	DPD	OPRT
Control	14.0	1.92	2.68
TRAIL (1 mg/kg)	12.0	1.68	2.93
S-1 (10 mg/kg)	12.9	1.57	2.75
TRAIL+ S-1	11.7	1.46	2.84

Expression relative to that of  $\beta$ -actin.

Table II. Protein levels of TS, DPD and OPRT in nude mouse tumors treated with TRAIL or S-1, alone and in combination.

Treatment	TS	DPD	OPRT
Control	414.6	88.0	11.7
TRAIL (1 mg/kg)	327.4	62.3	13.6
S-1 (10 mg/kg)	345.3	63.5	12.5
TRAIL+ S-1	253.7	59.9	13.1

ng/mg protein.

real-time RT-PCR. TS mRNA expression was reduced in TRAIL- or S-1-treated tumors, and even more reduced in TRAIL plus S-1-treated tumors. DPD mRNA expression was reduced in TRAIL-or S-1-treated tumors, and even more reduced in TRAIL plus S-1-treated tumors. OPRT mRNA expression was increased in S-1, TRAIL, and TRAIL plus S-1-treated tumors. In short, combined treatment of TRAIL and S-1 also induced

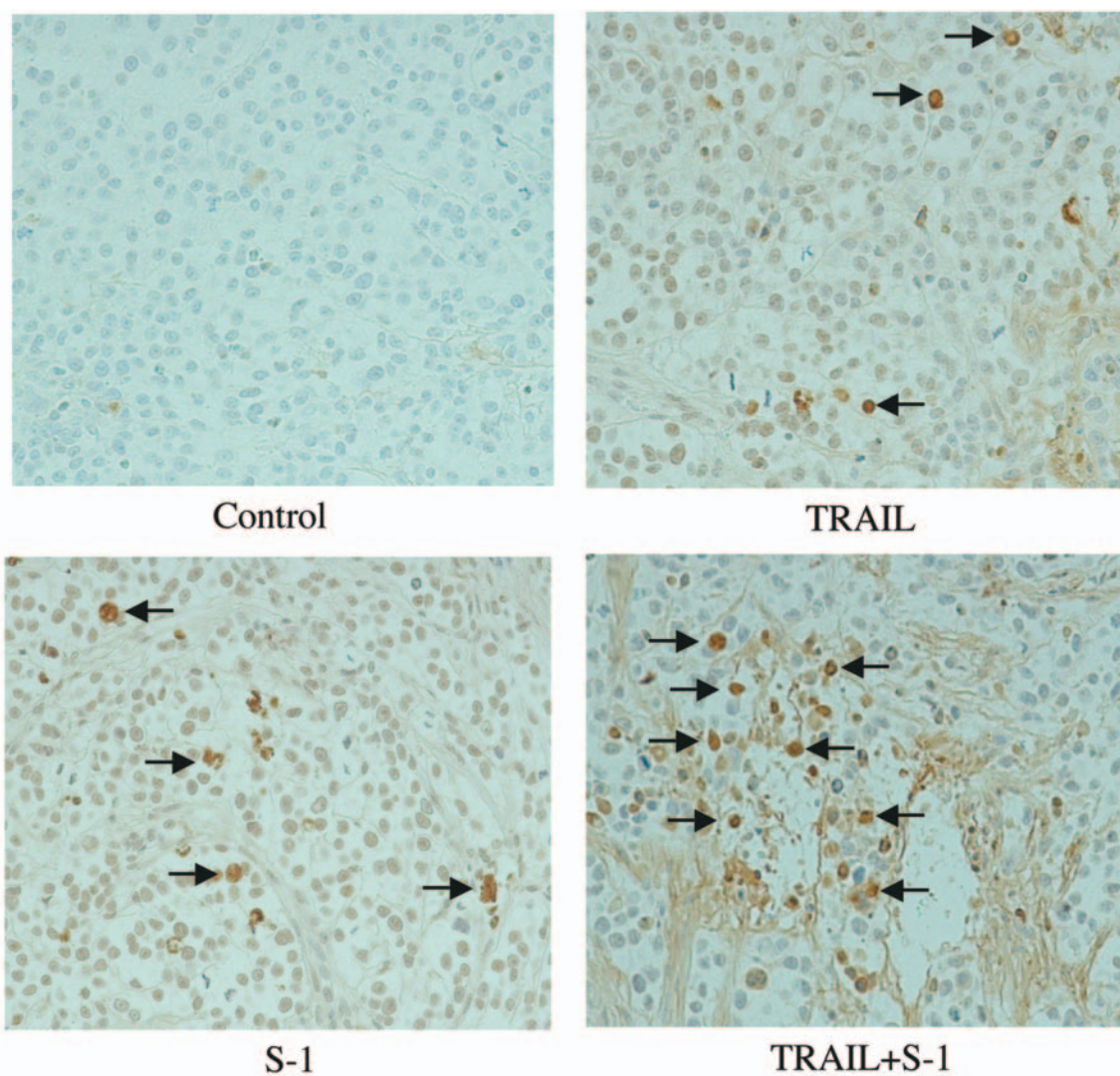


Figure 7. TUNEL assay. The largest number of apoptotic cells was seen in the tumors treated with the TRAIL and S-1 combination. Arrows show apoptotic cells (original magnification x300).

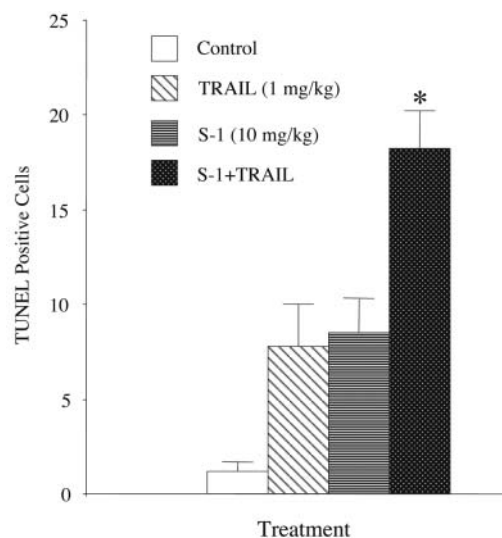


Figure 8. Analysis of the degree of apoptosis present in tumors with TRAIL alone, S-1 alone and TRAIL plus S-1. The TRAIL and S-1 treatment more markedly induced apoptosis than either agent alone (\* $p < 0.01$ ). Bars, SD.

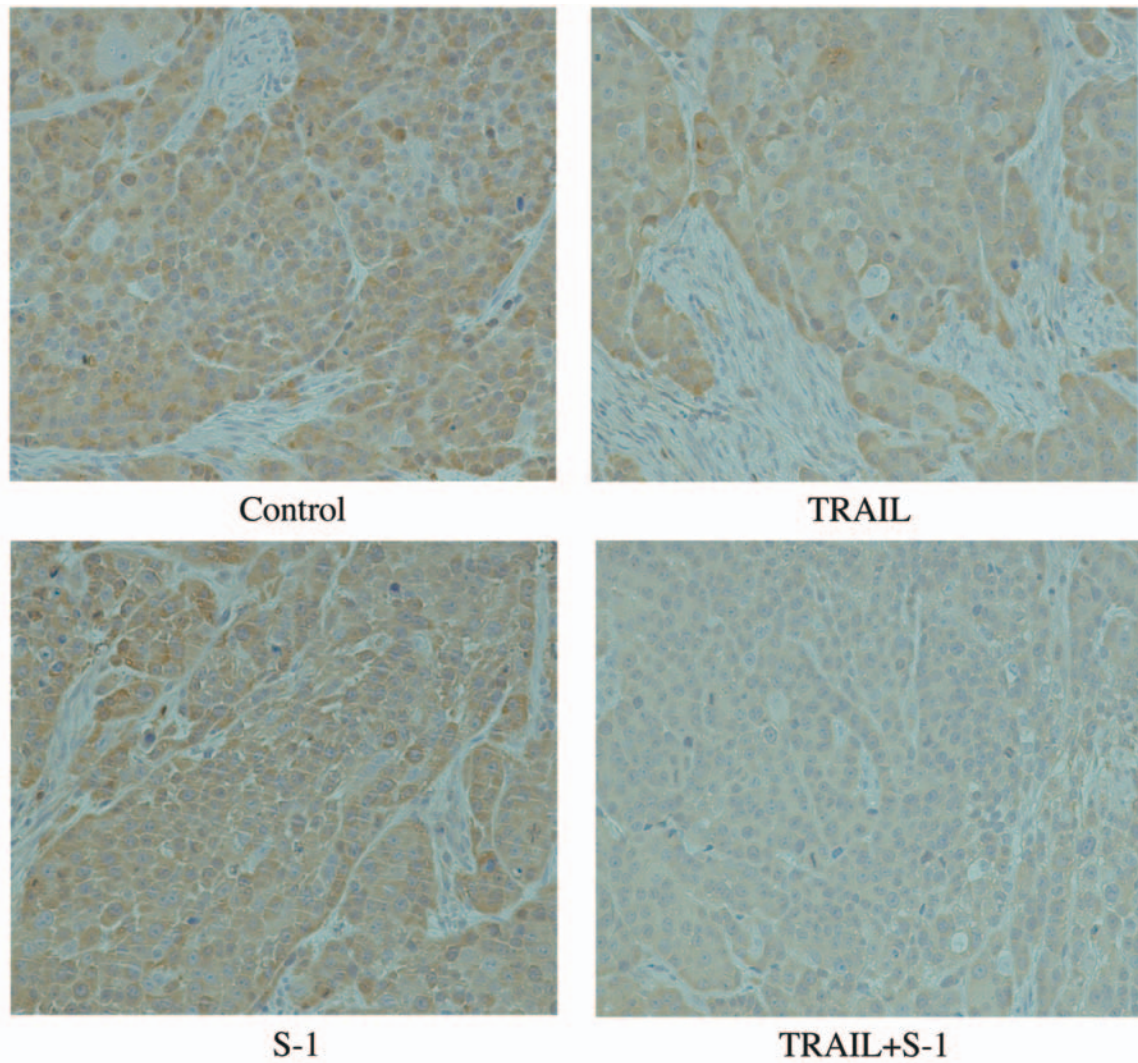


Figure 9. Expression of TS in nude mice tumors treated with TRAIL alone, S-1 alone and TRAIL plus S-1. TS expression was reduced slightly in TRAIL-treated tumors and markedly in TRAIL plus S-1 treated tumors.

down-regulation of TS mRNA and DPD mRNA expression, and the up-regulation of OPRT mRNA expression in B88 tumors similar to each protein expression level (Table I).

*Protein levels of TS, DPD and OPRT in nude mice tumor.*

We measured the protein levels of TS, DPD and OPRT in B88 tumors by ELISA. The TS protein level was lower in TRAIL-or S-1-treated tumors, and even more reduced in TRAIL plus S-1- treated tumors. The DPD protein level was lower in TRAIL- or S-1- treated tumors, and even more so in TRAIL plus S-1-treated-tumors. The OPRT protein level was higher in S-1, TRAIL and TRAIL plus S-1-treated tumors. Briefly, the combined treatment of TRAIL and S-1 induced the down-regulation of TS

protein and DPD protein, and the up-regulation of OPRT protein level in B88 tumors similar to each protein expression (Table II).

**Discussion**

OSCC, along with pharyngeal carcinoma, is the sixth most common cancer in the world (28). In addition, OSCC is a significant public health problem throughout the world because oral function is very important for breathing, eating and vocalizing. Moreover, OSCC is an aggressive human malignancy and prognosis is poor except for a few patients with early stage OSCC. Although the currently used combined modality therapies improved the outcome of patients with advanced OSCC, their efficacies are still

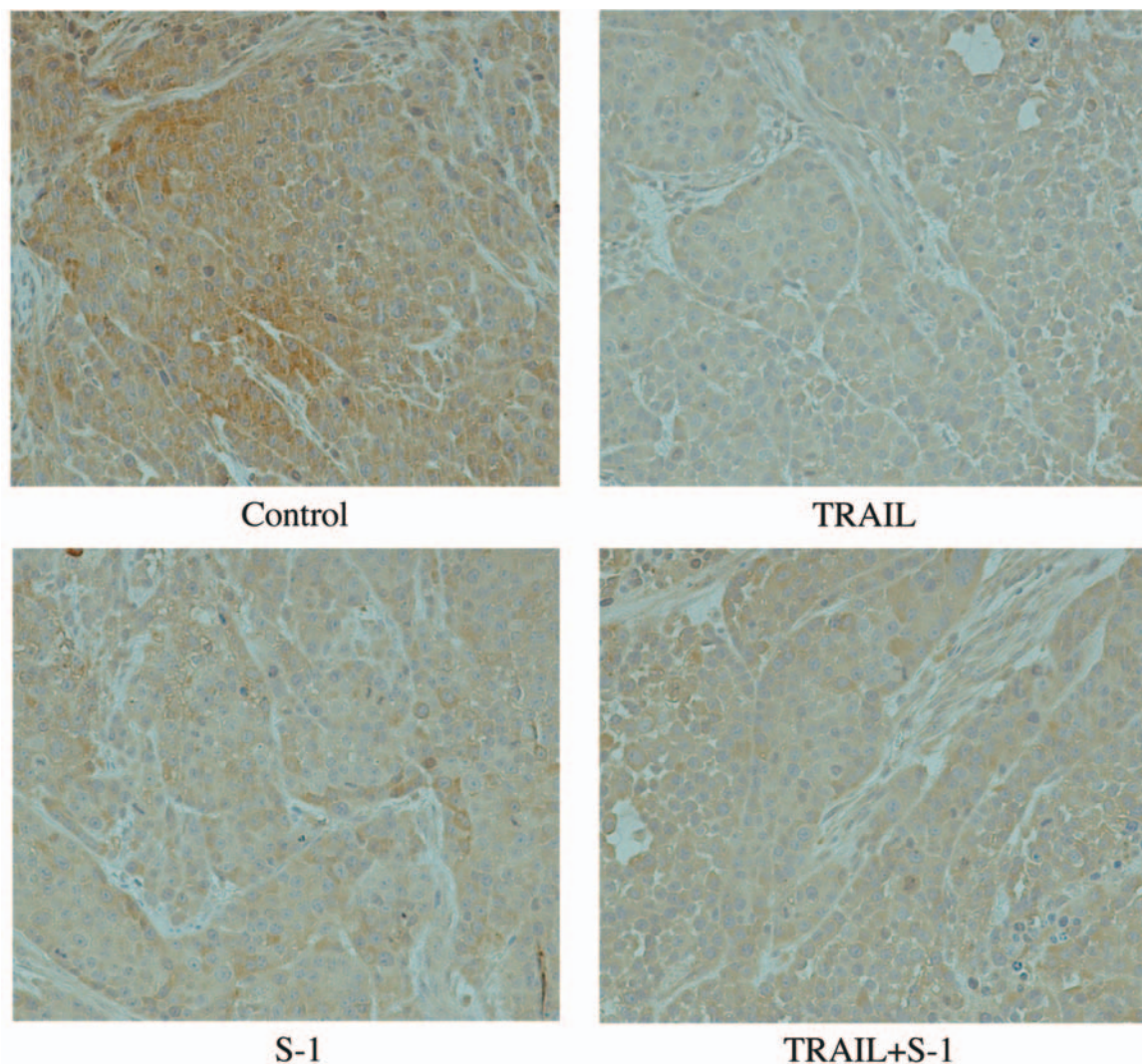


Figure 10. Expression of DPD in nude mice tumors treated with TRAIL alone, S-1 alone and TRAIL plus S-1. DPD expression was reduced in TRAIL, S-1 and TRAIL plus S-1-treated tumors.

limited and may cause serious side-effects or oral dysfunctions. Therefore, there is a general agreement for the need for 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 gastrointestinal carcinoma including OSCC were identified (29). The degree of TS, DPD or OPRT protein expression is thought to be one of most reliable factors of all. Much research has 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 led us to investigate the new therapeutic agents which can down-regulate TS and DPD expression, and up-regulate OPRT expression against OSCC cells.

Interestingly, Mizutani *et al.*, reported that a combination of TRAIL and 5-FU could overcome the resistance of renal cell carcinoma to TRAIL or 5-FU by down-regulating the expression of TS and DPD and up-regulating the expression of OPRT. This led us to believe that TRAIL might enhance the sensitivity of OSCC to S-1 by regulating the predictive factors of 5-FU. In addition, it has been reported that TRAIL can exert potent cytotoxic activity against many tumor cell lines, but not most normal cells (33, 34). In addition, TRAIL also suppressed tumor growth *in vivo* without affecting normal tissues in animal models (11, 35). If TRAIL can induce the chemosensitivity of S-1 to OSCC without serious side-effects, a combined therapy of TRAIL and S-1 should be useful for various malignant tumors as well as OSCC.

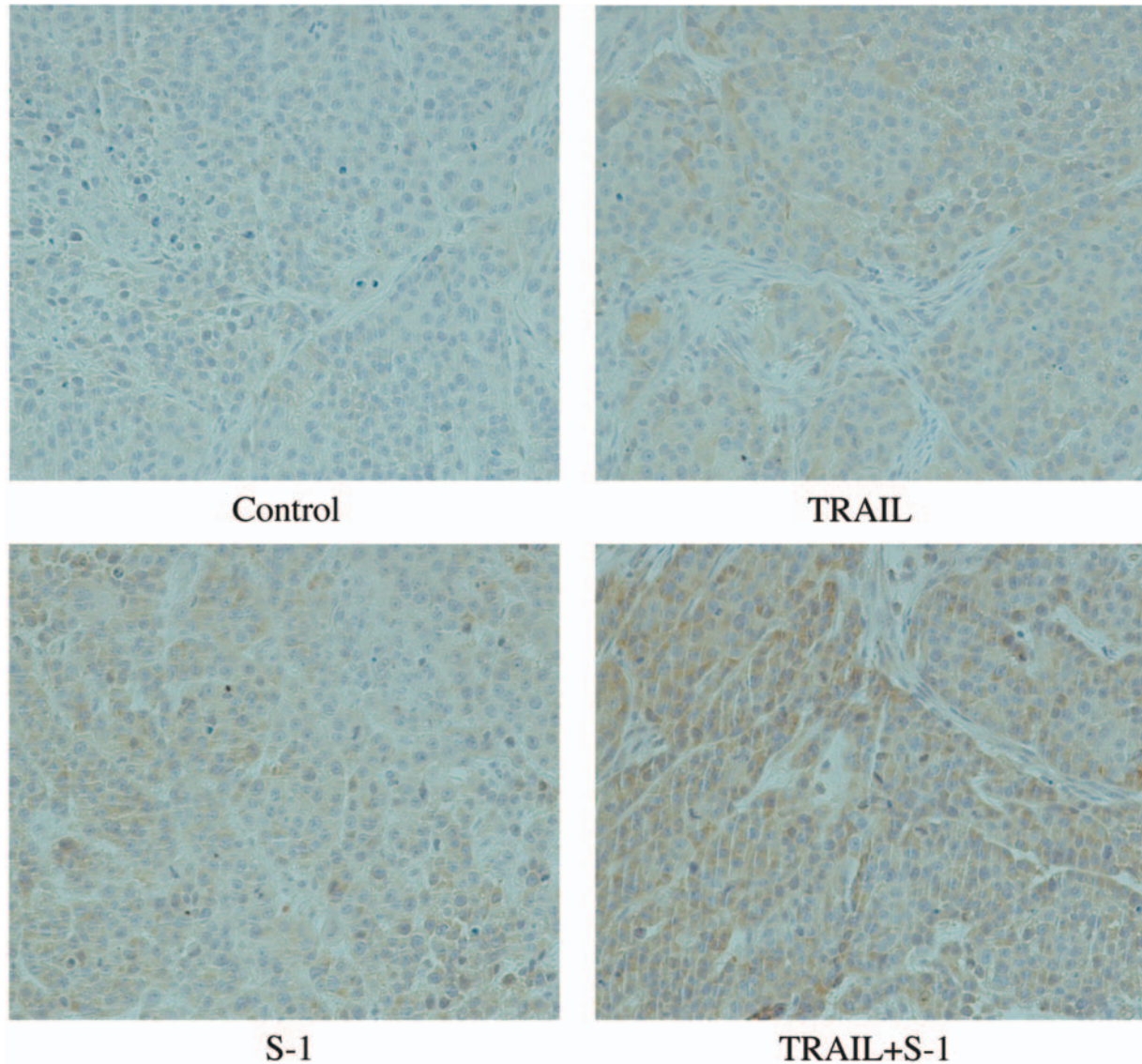


Figure 11. Expression of OPRT in nude mice tumors treated with TRAIL alone, S-1 alone and TRAIL plus S-1. OPRT expression was increased slightly in TRAIL-treated tumors and S-1 treated tumors, and markedly in TRAIL plus S-1-treated tumors.

In this study, we examined the response of human OSCC cells to TRAIL alone and in combination with S-1 using nude mice xenograft models. We also carried out an investigation of the mechanisms of the antitumor effects of TRAIL in combination with S-1. Our results demonstrated that TRAIL alone significantly inhibited the growth of B88 tumors when compared with that of controls. The antitumor activity of TRAIL 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 (Figures 3 and 4). Interestingly, combined therapy of TRAIL and S-1 as well as TRAIL alone was able to induce down-regulation

of TS and DPD expression and the up-regulation of OPRT expression both at the protein and mRNA level (Figures 5-7; Table I). Moreover, the combined therapy of TRAIL plus S-1 as well as TRAIL alone was able to induce the down-regulation of TS and DPD protein, and the up-regulation of OPRT protein (Table II). These findings suggest that combined therapy with TRAIL and S-1 as well as TRAIL alone may lead to the enhancement of chemosensitivity to S-1 in xenograft tumors. Combined therapy with TRAIL and S-1 may exert beneficial antitumor effects on these patients with advanced OSCC resistant to S-1. From this respect, combined therapy TRAIL and S-1 may have wide clinical application.

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*Received February 26, 2007*

*Revised May 22, 2007*

*Accepted May 24, 2007*