Efficacy of schedule-dependent metronomic S-1 chemotherapy in human oral squamous cell carcinoma cells

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Abstract. Metronomic chemotherapy is based on administration of anticancer agents at low-doses at close regular intervals with no prolonged breaks, and aims to inhibit vascular endothelial cells as well as tumor cells. Recently, it was suggested that metronomic chemotherapy exerts anti-angiogenic effects by inducing thrombospondin-1 (TSP-1) and early growth response-1 (EGR-1), and antitumor effects by suppressing cancer stem cells. S-1 is a novel orally administered anticancer drug that is a combination of tegafur, 5-chloro-2, 4-dihydroxypyridine and oteracil potassium for maintaining efficacious concentrations of 5-FU and reducing the serious gastrointestinal toxicity associated with 5-FU. In the present study, we tried to determine the suitable administration method of S-1 against oral squamous cell carcinoma as a metronomic chemotherapy. We performed *in vivo* experiments in which tumor-bearing nude mice were used to examine the antitumor activity of S-1 (6.9 mg/kg). HSC2 tumors were treated with three different regimens, given as 4-week treatment and 2-week rest (4W-2W, 1 cycle); 2-week treatment and 1-week rest (2W-1W, 2 cycles); or alternate days treatment (1D-1D, 6 weeks). A fourth group served as control. Antitumor effects and body weight changes were compared in each group. Expression of TSP-1, EGR-1, CD31 and CD44 in HSC2 tumors was examined by immunohistochemistry. The treated groups showed higher tumor growth inhibition compared to the control group, and the relative tumor growth inhibition was not different between the treated groups. Briefly, each relative tumor growth inhibition was 32.4% (4W-2W), 39.6% (2W-1W) and 37.0% (1D-1D). During treatment periods, body weights were lower in the mice with 4W-2W or 2W-1W than 1D-1D or control. Moreover, reduction of microvessel density and CD44 expression, and induction of TSP-1 and EGR-1 expression was markedly seen in 1D-1D-treated tumors compared to 4W-2W-, 2W-1W-treated tumors or untreated control tumors by immunohistochemistry. These findings suggest that the 1D-1D regimen is more useful than the 4W-2W or 2W-1W regimen as a metronomic chemotherapy.

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Introduction

Oral squamous cell carcinoma (OSCC), a subtype of head and neck cancer is the most common neoplasm of the oral cavity, which accounts for 90% of all diagnosed oral malignancies (1). OSCC constitutes the 8th most frequent cancer worldwide (2) and more than 300,000 new cases are diagnosed annually (3), whereas cancer of the head and neck accounts for approximately 643,000 new cases and 350,000 deaths per year (4,5). Over 85% of tumors of this region are epithelial tumors, especially squamous cell carcinomas (HNSCCs) (6). Chemoradiation after surgery is the standard treatment for patients with locally advanced HNSCC, but long-term survival in HNSCC patients is poor despite the recent advances in cancer therapy (7). Conventional chemotherapeutic modalities of OSCC/HNSCC consists of treating the patients with various drugs; e.g., cisplatin, fluorouracils, taxans, irinotecan (5) at their maximum tolerated doses (MTD), interrupted with long break periods between successive cycles. However, these chemotherapeutic regimens have a myriad of side-effects including neutropenia with increased rate of infection, diarrhea, renal failure, nausea, vomiting, dermatitis, and mucositis (5,8,9).

Among the fluorouracil-based drugs, S-1 (Taiho Pharmaceutical Co. Ltd., Tokyo, Japan) is potentially more active and less toxic than of 5-fluorouracil (5-FU) and reported to be effective against head and neck cancer with 34% response rate (8). S-1 consists of tegafur (prodrug of 5-FU), 5-chloro-2,4-dihydroxypyridine (CDHP; augments the activity of 5-FU by inhibiting DPD) and potassium oxonate (Oxo; reduces gastrointestinal toxicity by inhibiting 5-FU phosphorylation) at a molar ratio of 1:0.4:1 (10). There are numerous reports of S-1 clinical trials on HNSCC patients either alone or in combination with other drugs or radiotherapy (11-14), but high doses (60-80 mg/m^2) of S-1 causes both hematological and non-hematological toxicities in patients (8,9,11).

In contrast to the conventional MTD chemotherapy, metronomic chemotherapy, which refers to low-dose chemotherapy administered at a close regular intervals with no prolonged breaks (15) has shown effective antitumor efficacy by inhibiting tumor angiogenesis with reduced toxicity (16,17). Metronomic

low-dose chemotherapy with S-1 in combination with other drugs is reported to be effective in colorectal (10,18), hepatocellular (19) carcinoma mouse xenograft models, whereas metronomic tegafur-uracil (UFT) was effective in breast (20,21), gastrointestinal (22), ovarian cancer (23) and lung adenocarcinoma (24). Ooyama *et al* (10) have shown the efficacy of metronomic S-1 and capecitabine against human colon cancer xenografts and also confirmed the anti-angiogenic properties of S-1, suggesting that pharmacokinetics of S-1 might be similar to the concept of metronomic chemotherapy. There are few reports on metronomic chemotherapy with placitaxel (25), celecoxib and methotrexate (26) in oral/head and neck cancer. However, no preclinical/clinical reports on S-1 metronomic chemotherapy against OSCC/HNSCC are available. Therefore, rational strategies for developing new metronomic protocols and schedules with conventional drugs are necessary for gaining more favorable outcome of OSCC treatments.

The molecular and cellular mechanisms involved in growth, survival and expansion of solid tumors in HNSCC are not completely understood, but it is established that formation of new blood vessels (angiogenesis) and the co-option of existing vessels play a central role (27). Shaked *et al* (28) reported that, metronomic chemotherapy inhibits tumor angiogenesis and tumor growth by various mechanisms: i) killing endothelial cells through upregulation of anti-angiogenic factors [e.g. thrombospondin-1 (TSP-1)] and downregulation of pro-angiogenic factors [e.g. vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) or hypoxia-inducible factor-1 (HIF-1)]; ii) decreasing viability and mobilization of bone marrow-derived circulating endothelial progenitor cells (CEPs); iii) eradication and disruption of cancer stem cells (CSCs); iv) suppression of T regulatory cells; and v) killing the tumor cells directly (28-30). VEGF or VEGF-A is a key stimulator (10,31) of angiogenesis, whereas anti-angiogenic factors TSP-1 promotes endothelial cell apoptosis (10,32) and can suppress the mobilization of CEPs (28). Moreover, early growth response-1 (EGR-1) plays crucial role in angiogenesis by mediating the expression of TSP-1 (33), VEGF (34) and fibroblast growth factor-2 (FGF-2). Several reports on metronomic chemotherapy with S-1, placitaxel and other drugs in colorectal, hepatocellular, gastrointestinal and breast cancers have confirmed the association of downregulated VEGF expression and upregulated TSP-1 expression with the anti-angiogenic efficacy of metronomic chemotherapy (10,19,21,22,25,35).

Reports on a wide variety of cancers have demonstrated that only a distinct subpopulation of tumor cells, the CSCs, have the ability to undergo self-renewal and differentiation to initiate tumorigenesis and contribute to the recurrence and metastasis of cancers in humans (36,37). CSCs are also present in perivascular niches; release angiogenic factors in hypoxic conditions, and establish a permissive vascular niche in tumors (37). The molecular markers of CSC, which are most commonly used to detect tumor pathogenicity and angiogenesis in HNSCC are the cluster of differentiation (CD)34, CD133, CD24, CD44, CD29 and CD31 markers (38). In HNSCC, it has been shown that CD44-positive cancer cells can initiate *in vivo* tumor formation and have increased resistance to drug and radiation therapies. CD44 overexpression is also associated with poor prognosis (39,40). Moreover, Hollemann *et al* (27) demonstrated new vessel formation can be detected by the endothelial marker CD31 in HNSCC. Therefore, CD31 and CD44 can be used to evaluate the degree of tumor angiogenesis and can act as prognostic marker of OSCC (39-42).

In this study, we used OSCC xenograft models to clarify the suitable administration method of S-1 against oral squamous cell carcinoma as a metronomic chemotherapy. We also checked the efficacy of metronomic 5-FU against OSCC *in vitro*. In addition, we investigated the mechanisms of the antitumor and anti-angiogenic effects of metronomic S-1 chemotherapy by evaluating the expression of VEGF, TSP-1 and EGR-1. The effect of metronomic S-1 on the suppression of CSC in tumors was also evaluated by detecting the expression pattern of CD44 and CD31. Moreover, we quantified microvessel density in tumors.

Materials and methods

OSCC cell line and nude mice. Human tongue squamous cell carcinoma (HSC2) cell line was purchased from Cell Bank, RIKEN BioResource Center (Ibaraki, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 μg/ml streptomycin, 100 U/ml penicillin (Invitrogen) in a humidified atmosphere containing 5% CO_2. Four-week-old female CAnN. Cg-Foxnlnu/CrlCrlj athymic nude mice (average weight 15.0 g) were purchased from CLEA Japan Co. Ltd. (Tokyo, Japan). The mice were maintained under specific-pathogen-free conditions and provided with sterile water and food *ad libitum*. All manipulations were carried out aseptically inside a laminar flow hood. The mice were maintained and handled in accordance with the Guidelines for Animal Experimentation of Yamaguchi University.

Examination of antitumor activity of metronomic S-1 in vivo. HSC2 cells ($1x10^6$) were suspended in 0.1 ml of serum-free medium and injected into the subcutaneous tissue of 5-weeks-old mice using a 27-gauge needle. When the estimated tumor volume (0.5 x length x width^2) reached 100-150 mm^3, the tumor-bearing mice were randomly divided into one control and three treatment groups (5 mice/group). HSC2 tumors were treated with three different regimens of S-1 (6.9 mg/kg) dissolved in 0.5% hydroxypropyl methylcellulose (HPMC). The treatment groups were: group 1 (4W-2W; 1 cycle); S-1 administered for four weeks with 2-week rest, group 2 (2W-1W; 2 cycles); 2-week treatment with S-1 and 1-week rest, and group 3 (1D-1D; 6 weeks); treatment in alternate days with S-1. HPMC (0.5%) was administered to group 4, the control group (Fig. 1). Tumor size and body weight were monitored and measured twice/week. All mice were sacrificed at the end of each treatment regimen. Antitumor effects and body weight changes were compared among the groups.

Immunohistochemical assay for EGR-1, TSP-1, VEGF, CD44 and CD31. HSC2 tumors harvested at autopsy were embedded in paraffin blocks. Four-micrometer-thick sections were prepared from the blocks and mounted on slides. These sections were fixed and then processed for immunostaining using the anti-EGR-1 mouse monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-TSP-1 mouse monoclonal antibody (Santa Cruz), anti-VEGF mouse monoclonal antibody

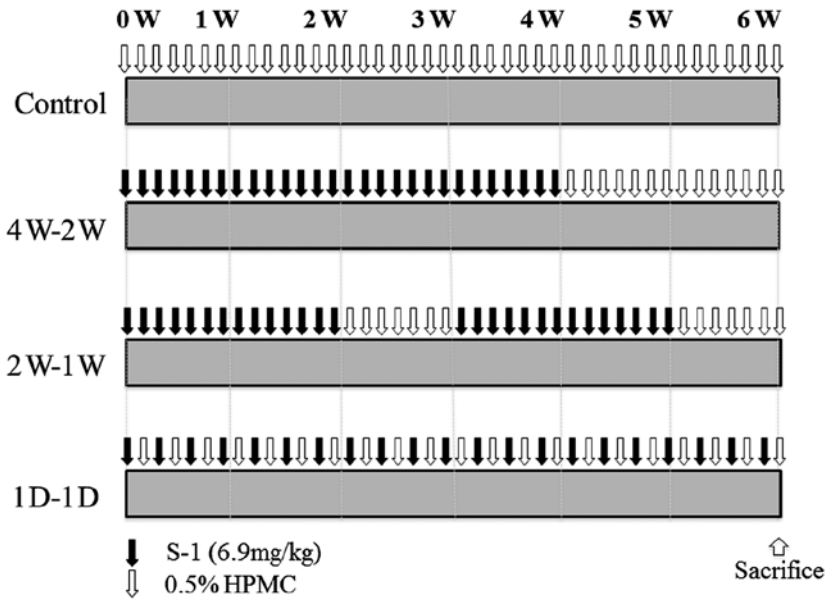

Figure 1. The treatment regimens and administration schedules for the *in vivo* tumor growth assay. The control group was injected with 0.5% HPMC only for 6 weeks. The treatment regimens were as follows: 4W-2W (1 cycle), S-1 (6.9 mg/kg) administered for 4 weeks with 2-week rest; 2W-1W (2 cycles); 2-week treatment with S-1 (6.9 mg/kg) and 1-week rest; and 1D-1D (1 cycle); treatment in alternate days with S-1 (6.9 mg/kg) for 6 weeks.

(Santa Cruz), anti-HCAM (CD44) mouse monoclonal antibody (Santa Cruz), or anti-PCAM (CD31) rabbit polyclonal antibody (Santa Cruz), and appropriate peroxidase conjugated anti-rabbit or mouse IgG second antibody. Negative controls were done using non-specific IgG. The blocking and immunostaining were performed using Dako EnVision kit (Dako, Glostrup, Denmark). All the specimens were counterstained with hematoxylin. The slides were then examined under a bright-field microscope. A positive reaction was detected in the cytoplasm or nucleus as reddish-brown precipitates. For quantification of microvessel density (MVD), CD31-positive vessels were counted in randomly selected 10 areas per tumor in each treatment group at 200-fold magnification.

Examination of antitumor activity of metronomic 5-FU in vitro. HSC2 cells ($5x10^3$ cells per well) were seeded on 96-well plates (Becton-Dickinson Labware, Franklin Lakes, NJ, USA) in DMEM supplemented with 10% FBS. Twenty-four hours later, the cells were treated with four different regimens using 0.5 μg/ml 5-FU. The treatment regimens were: 96-h treatment and 48-h rest (96 h-48 h, 1 cycle); the 48-h treatment and 24-h rest (48 h-24 h; 2 cycles); the 24-h treatment and 12-h rest (24 h-12 h; 4 cycles); and the 16-h treatment and 8-h rest (16 h-8 h; 6 cycles). A fifth group served as control (Fig. 5). At the end of each treatment, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well (25 μl/well) of the 96-well plate and incubated for 4 h. The blue dye taken up by cells was dissolved in dimethyl sulfoxide (100 μl/well), and the absorbance was measured with a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) at 490 nm. Growth inhibitory effects were compared among the groups. All assays were run in triplicate.

Western blot analysis for TSP-1, EGR-1, VEGF and CD44 in vitro. Control and 5-FU-treated HSC2 cells were lysed with RIPA Buffer (Thermo Scientific, Kanagawa, Japan). Whole cell lysates containing 50 μg protein/sample were subjected to electrophoresis on 10% SDS-polyacrylamide gels, and then transferred to a PVDF membrane. The membranes were blocked and incubated with the anti-EGR-1 mouse monoclonal antibody (Santa Cruz), anti-TSP-1 mouse monoclonal antibody (Santa Cruz), anti-VEGF mouse monoclonal antibody (Santa Cruz), or anti-HCAM (CD44) mouse monoclonal antibody (Santa Cruz). All antibodies were detected using Western Breeze chromogenic immunodetection system (Invitrogen) according to the manufacturer's instructions. Also, anti-α-tubulin monoclonal antibody (Santa Cruz) was used for normalization of western blot analysis.

Statistics. The significance of the *in vivo* and *in vitro* results was determined by the Mann-Whitney U test or one-way ANOVA. The differences were considered statistically significant at $P<0.05$.

Results

Evaluation of the antitumor effect of metronomic S-1 chemotherapy in vivo. An *in vivo* experiment was carried out with HSC2 tumor-bearing nude mice to examine the antitumor activity of four different treatment regimens of low dose metronomic S-1 (6.9 mg/kg). The treatment regimens and schedules are showed in Fig. 1. As shown in Fig. 2A, all treated-groups showed significant tumor growth inhibition compared to the control group ($P<0.01$). However, the relative tumor growth inhibition was not significantly different between the treated groups. Briefly, each relative tumor growth inhibition was 32.4% (4W-2W), 39.6% (2W-1W) and 37.0% (1D-1D). Neither treatment regimen induced a significant body weight loss during treatment periods. However, body weights were lower in the mice with 4W-2W or 2W-1W, than 1D-1D or control group (Fig. 2B).

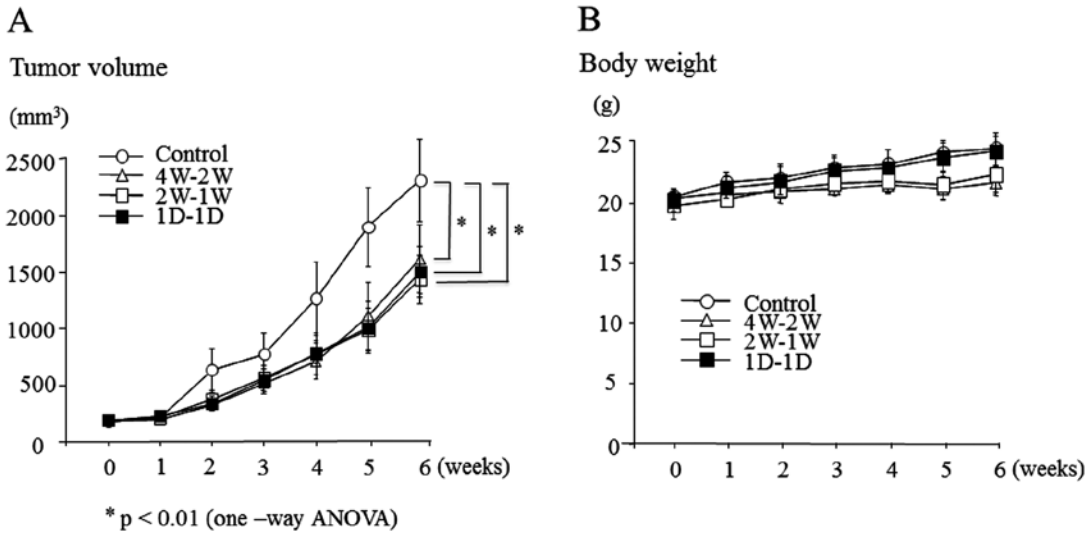

Figure 2. Anti-tumor effects of metronomic S-1 *in vivo*. HSC2 cells ($1x10^6$ cells) were suspended in 0.1 ml of serum-free medium and injected into the subcutaneous tissue of nude mice. The tumors were treated with S-1 (6.9 mg/kg) according to the schedule shown in Fig. 1. (A) Tumor volumes of the control and S-1-treated nude mice. Tumor volume was determined by the formula: 0.5 x length x $width^2$. The values shown are the mean of five tumors (mm^3); bars, SD. $^*p<0.001$ when compared with that of control by one-way ANOVA. (B) The body weight of the control and S-1-treated nude mice. The values shown are the mean body weight (g) of five mice; bars, SD.

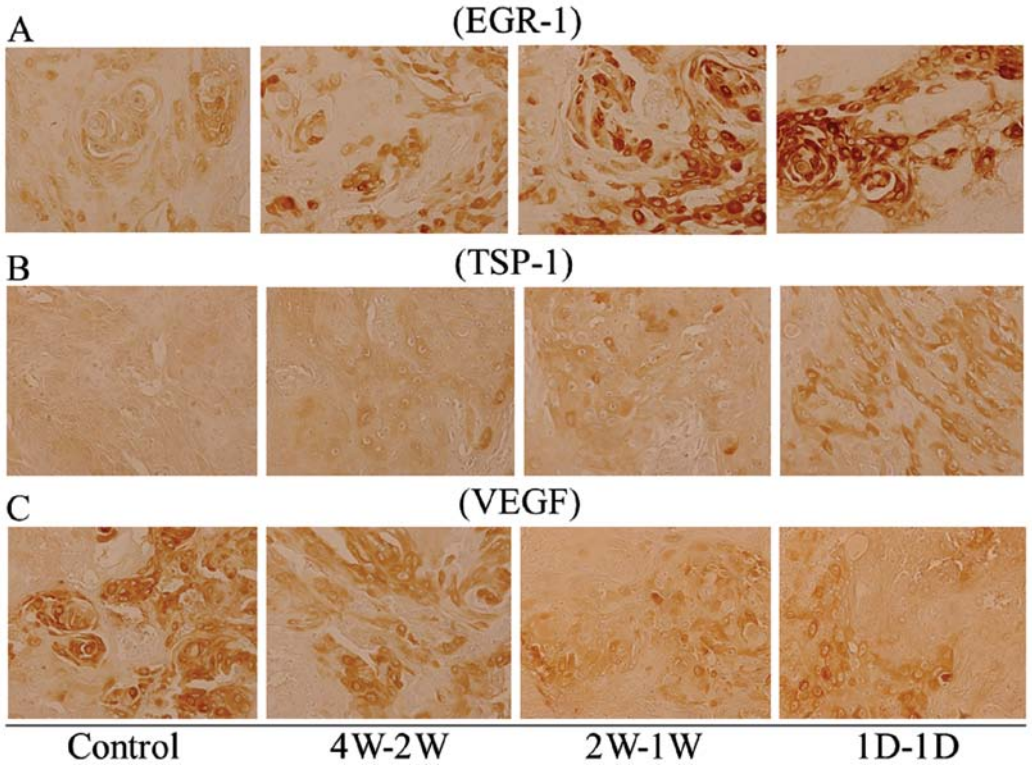

Figure 3. Expression of (A) EGR-1, (B) TSP-1 and (C) VEGF protein in HSC2 tumors. Tumors were either untreated (control) or treated with different treatment regimens of S-1 (6.9 mg/kg). The expression of EGR-1, TSP-1 and VEGF was detected with immunohistochemical staining. Dark brown staining on the nuclei is positive (original magnification, x200).

Evaluation of expression of EGR-1, TSP-1, VEGF, CD44 and CD31 in tumor tissues in vivo by immunohistochemistry and quantification of MVD. We carried out immunohistochemistry experiments to evaluate the expression pattern of angiogenic and anti-angiogenic factors in mouse tumors treated with metronomic S-1. The expression of anti-angiogenic factors, TSP-1 and EGR-1 was markedly induced in 1D-1D-treated tumors compared to 4W-2W-, 2W-1W-treated tumors or untreated control tumors (Fig. 3A and B). The expression level of VEGF, a key angiogenic factor was also

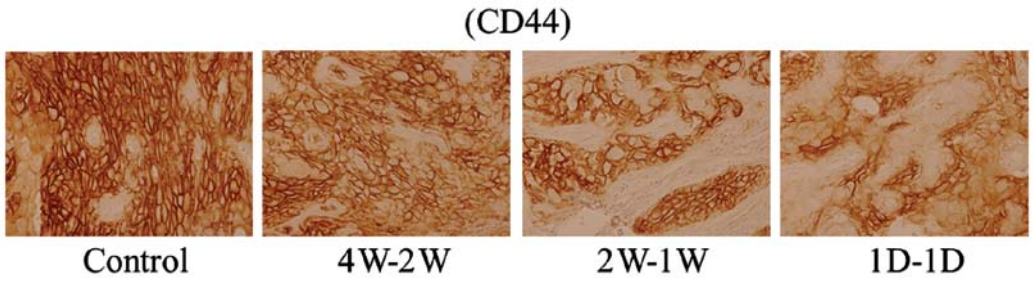

Figure 4. Expression of stem cell factor CD44 in HSC2 tumors detected with immunohistochemical staining. Tumors were either untreated (control) or treated with different treatment regimens of S-1 (6.9 mg/kg). Dark reddish-brown staining on the nuclei is positive (original magnification, x200).

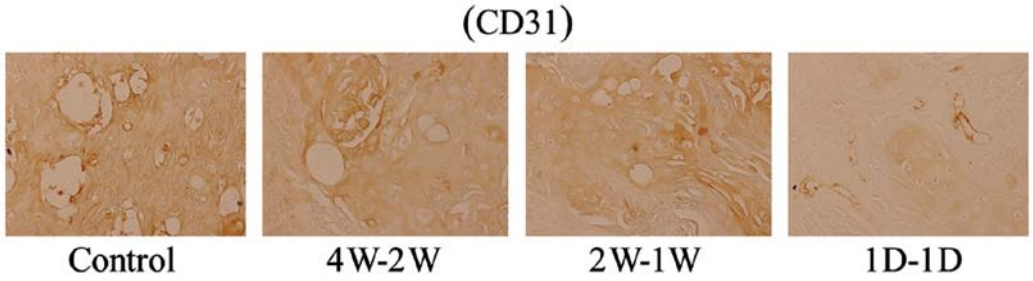

Figure 5. Expression of endothelial marker CD31 in HSC2 tumors detected with immunohistochemical staining. Tumors were either untreated (control) or treated with different treatment regimens of S-1 (6.9 mg/kg). Dark reddish-brown staining on the nuclei is positive (original magnification, x200).

evaluated. A significant reduction of VEGF expression was observed in 1D-1D-treated mouse tumors than 4W-2W, 2W-1W or untreated control (Fig. 3C). Moreover, in order to examine the efficacy of metronomic S-1 in the suppression of CSCs, the expression of cancer stem cell factor CD44 was evaluated. Downregulated expression of CD44 was observed in treated mouse tumors compared with the untreated control, with 1D-1D-treated tumors showing the most reduced expression of CD44 compared with 4W-2W- and 2W-1W-treated tumors (Fig. 4). Expression of endothelial marker CD31 was also decreased in all treatment groups compared with the untreated control, with marked reduction of CD31 expression in 1D-1D-treated tumors (Fig. 5).

Accordingly, MVD was significantly reduced in all three treated groups compared with the control (P<0.01). However, reduction of MVD was significantly higher in 1D-1D treatment group compared with the other treatment groups (Table I).

Evaluation of growth inhibitory effect of metronomic 5-FU in vitro. To further examine the efficacy of metronomic S-1 chemotherapy against HSC2 cells, we investigated the growth inhibitory effect of low dose metronomic 5-FU (0.5 μg/ml) *in vitro*. HSC2 cells were treated with four different treatment regimens as shown in Fig. 6. As observed by MTT assay, 96 h-48 h, 48 h-24 h, 24 h-12 h and 16 h-8 h treatment groups showed significant (P<0.01) growth inhibition of HSC2 cells compared with the untreated control (Fig. 7A). There was no significant difference among the treatment groups. However, the highest inhibition of cell growth was detected in 16 h-8 h treatment group (Fig. 7A).

Table I. Microvessel density in tumors.

Treatment	Vessels
Control	14.6±3.75
4W-2W	8.80±1.32[a]
2W-1W	6.90±1.45[a]
1D-1D	3.40±1.51[a,b]

[a]p<0.01 Student's t-test; [b]p<0.05, 2W-1W vs. 2D-1D (Student's t-test).

Evaluation of expression of EGR-1, TSP-1, VEGF and CD44 in vitro by western blot analysis. Western blot analysis results in Fig. 7B show the expression of EGR-1, TSP-1, VEGF and CD44 in treated tumor tissues. The expression level of EGR-1 was relatively upregulated in 48 h-24 h, 24 h-12 h and 16 h-8 h treatment groups compared to the control. Moreover, compared with the control, the expression of TSP-1 was increased markedly in all four treatment groups (96 h-48 h, 48 h-24 h, 24 h-12 h and 16 h-8 h). In 16 h-8 h group, the expression of TSP-1 was highest which was approximately 3- to 4-fold increased expression to that of control (Fig. 7B). In contrast to EGR-1 and TSP-1 expression, VEGF expression was considerably downregulated in 24 h-12 h and 16 h-8 h treatment groups compared with 96 h-48 h, 48 h-24 h treatment groups or control. Similarly, the expression of cancer stem factor CD44 decreased slightly in 96 h-48 h, 48 h-24 h treatment groups compared with the control, while 24 h-12 h and 16 h-8 h treatment groups showed significantly reduced expression of CD44. Among all the

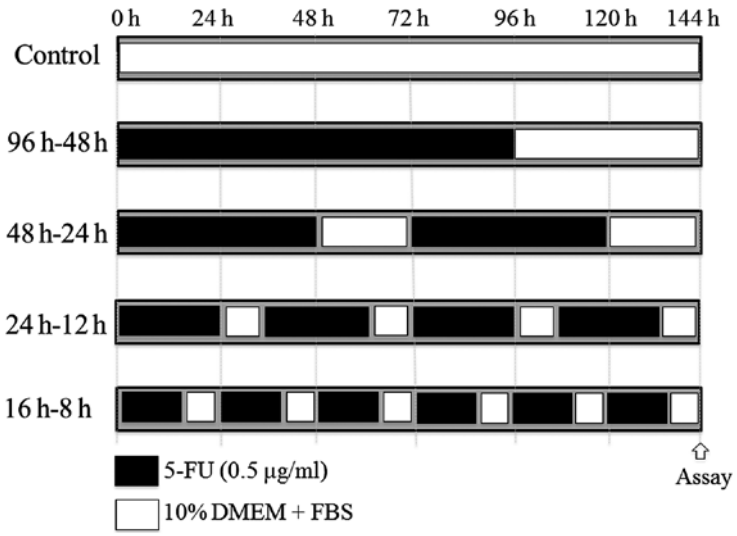

Figure 6. The treatment regimens and administration schedules for *in vitro* experiments. The control group was cultured in 10% DMEM and FBS, without 5-FU for 144 h. The treatment regimens were as follows: 96 h-48 h (1 cycle), 96-h treatment with 0.5 μg/ml 5-FU and 48-h rest; 48 h-24 h (2 cycles), 48-h treatment with 0.5 μg/ml 5-FU and 24-h rest; 24 h-12 h (4 cycles), 24-h treatment with 0.5 μg/ml 5-FU and 12-h rest; and 16 h-8 h (6 cycles), 16-h treatment with 0.5 μg/ml 5-FU and 8-h rest.

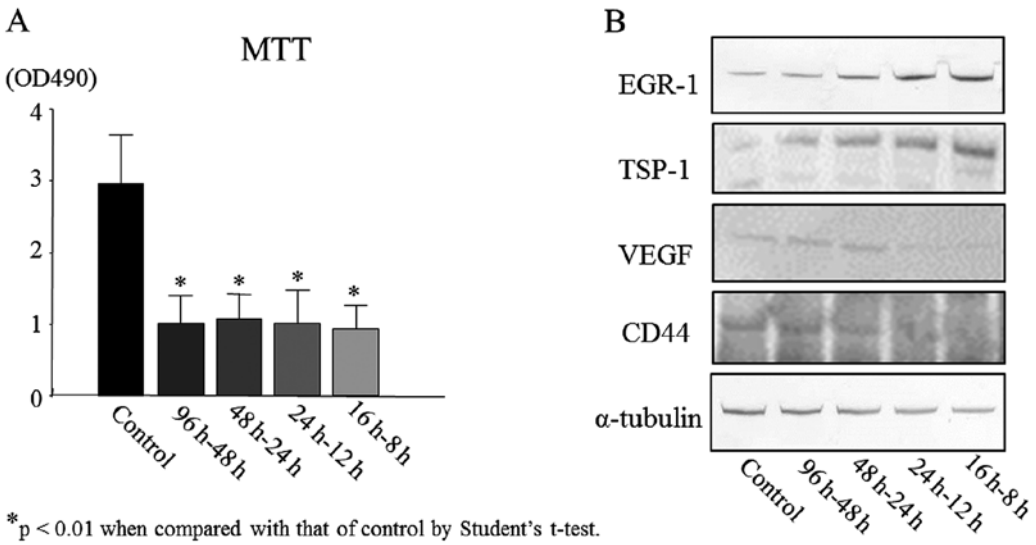

Figure 7. Anti-tumor effects of metronomic 5-FU *in vitro*. (A) Growth inhibitory effect of 0.5 μg/ml 5-FU on HSC2 cells. HSC2 cells ($5x10^3$ cells per well) were seeded on 96-well plates and exposed to 5-FU according to the schedule shown in Fig. 6. At the end of each treatment regimen, cells were counted using a microplate reader. Each data point represents the mean of three independent determinations. $^*p<0.05$ when compared with that of control by one-way ANOVA. (B) Expression of EGR-1, TSP-1, VEGF and CD44 protein in HSC2 cell lysates. HSC2 cells were treated according to the schedule shown in Fig. 6, and the whole cell lysates were obtained. Equal amount of cellular protein (50 μg) was subjected to SDS-PAGE, followed by western blot analysis for EGR-1, TSP-1, VEGF and CD44. α-tubulin was used as a control for equal protein load.

treatment groups, the 16 h-8 h treatment group showed highest expression of EGR-1 and TSP-1, and lowest expression of VEGF and CD44 compared to control (Fig. 7B).

Discussion

In our present study, we evaluated the efficacy of metronomic chemotherapy with low dose S-1 against OSCC both *in vivo* and *in vitro*. Metronomic chemotherapy, which refers to the administration of comparatively low doses of drugs on a frequent schedule, with no extended interruptions lacks the acute toxicity of conventional MTD chemotherapy, but effective even in low doses against tumor growth and expansion because it inhibits angiogenesis and decreases nutrition supply in tumors (16,22). The anti-angiogenic efficacy of metronomic chemotherapy is reported to increase when it is administered in combination with specific anti-angiogenic drugs and molecularly targeted therapies (23,24). Metronomic chemotherapy can be administered orally and is quite effective as adjuvant chemotherapy (16,20,24). Being less toxic, it is effective in palliative care (22,24) and can ensure normal oral functions in OSCC patients. Moreover, because of its anti-angiogenic

property, metronomic chemotherapy might not induce drug-resistance in tumors (32,43). Iwamoto *et al* (19) reported that hepatocellular tumor cell line that was intrinsically resistant to high concentration of fluorouracils *in vitro*, responds well to metronomic low dose S-1.

There are pre-clinical and clinical reports on the efficacy and anti-angiogenic property of metronomic S-1 and UFT against various cancers (11,19-25). Kato *et al* (24) reported a beneficial effect of low dose UFT as adjuvant chemotherapy in 999 lung adenocarcinoma (stage I) patients. In that study, UFT (250 mg/m^2/day) was administered twice daily for two years, which significantly improved overall survival of the patients with grade II adverse effects/toxicity in only 2% of the patients. Kato *et al* (24) also stated that, as fluorouracil is not a dose-dependent but a time-dependent drug and acts as anti-angiogenic agent, daily, low-dose, long-term administration of uracil-tegafur might be effective. Ooyama *et al* (10) and Iwamoto *et al* (19) have reported beneficial effects of metronomic S-1 in combination with anti-angiogenic agents against colon and hepatocellular cancer xenografts. Placitaxel is also effective as metronomic chemotherapy (25,44). A study conducted with 722 metastatic breast cancer patients who received 90 mg of paclitaxel/m^2 for 3 days/4 weeks with 10 mg of bevacizumab reported significantly prolonged progression-free survival of the patients with minimal toxicity (44).

Our results suggest that, administration of metronomic 6.9 mg/kg S-1 in alternate days (1D-1D) for 6 weeks is effective against OSCC tumors than treatment regimens with long intervals (4W-2W or 2W-1W), as 1D-1D markedly exert anti-angiogenic effects and reduce the expression of cancer stem cell markers. In our study, we chose S-1 over UFT because administration of S-1 in cancer patients often resulted in high concentration of 5-FU in blood and tumor tissue for long-term periods due to its biochemical modulation (19). Thus, S-1 might have stronger antitumor effect than UFT. Moreover, previous reports on nude mice xenograft experiments have confirmed the efficacy of S-1 in OSCC tumor growth inhibition, apoptosis and angiogenesis *in vivo* (45,46).

According to literature, metronomic chemotherapy inhibits tumor growth by targeting genetically stable endothelial cells within the vascular bed in tumors, upregulating anti-angiogenic factors (i.e. TSP-1, EGR-1) and downregulating angiogenic factors (i.e. VEGF), killing or disrupting pro-angiogenic bone marrow-derived cells, CEPs and CSCs (16,28-30). In our study, metronomic S-1, especially 1D-1D upregulated anti-angiogenic factors, TSP-1 and EGR-1 in mouse tumors. There are several reports that describe similar expression pattern of TSP-1 and EGR-1 in colorectal, gastrointestinal, hepatocellular cancer after administration of S-1, UFT or 5-FU (10,19,22,47). Allegrini *et al* (22) and Caballero *et al* (25) reported reduced expression of VEGF in gastrointestinal cancer treated with metronomic UFT and in HNSCC treated with metronomic placitaxel, respectively. We also observed downregulated expression of VEGF in all treatment groups of metronomic S-1, but the expression was reduced more markedly in tumors treated with 1D-1D. However, this finding contradicts with the results of Ooyama *et al* (10) and Iwamoto *et al* (19), as they reported no significant difference of VEGF expression between control and metronomic S-1-treated tumors. Bancroft *et al* (48) have shown that, expression of VEGF in HNSCC cell lines is associated with co-activation of both nuclear factor (NF)-κB and activator protein-1 (AP-1). Since 5-FU and S-1 are reported to suppress the expression of NF-κB (49,50) and VEGF (50) in OSCC cell lines, metronomic S-1 doses are expected to deliver similar results.

There are several factors other than TSP-1 and EGR-1, which act as inhibitors of angiogenesis, i.e. interferons, TGF-β, angiostatin, and endostatin. Moreover, VEGF is the major contributor of angiogenesis, and that bFGF, FGF, angiogenin, hepatocyte growth factor (HGF) and interleukin 8 (IL-8) is also known as stimulator of angiogenesis. In this study, we examined the expression of VEGF, TSP-1 and EGR-1 only. Further studies with other angiogenic and anti-angiogenic factors might help to clarify the efficacy and anti-angiogenic property of metronomic S-1 against OSCC.

We also examined the expression of cancer stem cell marker CD44, endothelial marker CD31, and MVD in tumors. Ooyama *et al* (10) and Iwamoto *et al* (19) showed downregulated CD31 expression and reduced number of microvessels in colon and hepatocellular tumors after treatment with metronomic S-1. Similarly, in our *in vivo* study, metronomic S-1 notably downregualted the expression of CD44 and CD31, and significantly reduced MVD in 1D-1D compared to the other treatment groups.

Moreover, in order to clarify the efficacy of metronomic S-1 *in vitro*, we carried out growth inhibition assay with metronomic, low dose 5-FU (0.5 μg/ml) and evaluated the expression of TSP-1, EGR-1, VEGF and CD44 in 5-FU-treated OSCC cells. In our *in vitro* study, metronomic 5-FU treatment regimens significantly inhibited OSCC tumor growth compared with the control group. Although, growth inhibition pattern of tumors were almost same among the 5-FU treatment regimens, we concluded that 16-h treatment and 8-h rest regimen (16 h-8 h; 6 cycles) is more effective against OSCC since it noticeably induced the expression of anti-angiogenic factors TSP-1 and EGR, and reduced the expression of angiogenic factor VEGF, and cancer stem cell marker CD44. Our experiment results *in vitro* regarding the expression of TSP-1. EGR-1, VEGF and CD44 coincided with our *in vivo* data with metronomic S-1.

Preclinical and clinical evidence suggests that, a drug that is administered at a specific point of the circadian clock of a patient which is designed to target specific cell cycle events or angiogenesis might be more effective against tumor progression and can achieve patient's maximum tolerance towards the toxicity of the drug (51). Chronomodulated delivery of fluorouracil at the early part of the resting period allowed better control of the plasma concentrations of the drug and showed less toxicity in patients (51). Therefore, close attention on designing the drug delivery scheduling of metronomic chemotherapy will accelerate favorable outcome of the patients.

In the present study, although the different scheduling of metronomic S-1 delivery did not show significant difference in tumor growth both *in vivo* and *in vitro*, schedules with more frequent drug administration and shorter resting period (1D-1D, 16 h-8 h treatment regimens) was most effective against OSCC as it induced the expression of anti-angiogenic factors, downregulated the expression of angiogenic factors and suppressed the cancer stem cell markers. In future, *in vivo* toxicological study will be carried out to investigate the possible adverse effects of metronomic S-1 among the treatment groups.

In conclusion, we have demonstrated the efficacy of metronomic low dose S-1 and 5-FU against OSCC preclinically. Our data suggests that, the efficacy of metronomic S-1 chemotherapy not only lies on the cytotoxic effect of S-1, but also its inhibitory action on angiogenesis and suppression of cancer stem cells. Therefore, metronomic S-1 might be a promising strategy for OSCC treatment. Further studies with metronomic S-1 in combination with various anti-angiogenic agents (sorafenib, bevacizumab, irinotecan, semaxinib, sunitinib) might deliver more promising results for the treatment of oral cancer.

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