The therapeutic potential of epigallocatechin-3-gallate against human oral squamous cell carcinoma through inhibition of cell proliferation and induction of apoptosis: *In vitro* and *in vivo* murine xenograft study

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Received October 19, 2018; Accepted May 10, 2019

DOI: 10.3892/mmr.2019.10331

Abstract. Oral squamous cell carcinoma (OSCC) is one of the most common malignant tumors in the oral region. Despite current therapeutic strategies, the survival rate has not been improved for several decades. Thus, it is important to develop a novel approach for the treatment of OSCC. Epigallocatechin-3-gallate (EGCG) is a major constituent of green tea and has previously been demonstrated to inhibit the growth of several types of cancer cells. However, few studies have investigated the effect of EGCG on human OSCC cells, especially in experimental animal models. The aim of the present study was to evaluate the therapeutic potential of EGCG for targeting human OSCC *in vitro* and *in vivo*. In the *in vitro* experiments, EGCG suppressed HSC-3 cell viability in a time- and dose-dependent manner. Cell cycle analysis revealed that EGCG induced G1 phase arrest of the tumor cells. Apoptosis was examined by Annexin V and propidium iodide staining, assays of caspase-3 and -7 activity and TdT-mediated dUTP nick end labeling (TUNEL) staining. Treatment with EGCG significantly increased caspase-3 and -7 activities, and the percentage of apoptotic cells when compared with control cells. In the *in vivo* xenograft experiment on mice, EGCG treatment resulted in a 45.2% reduction in tumor size as compared with the control group without weight loss. *In vivo* cell proliferation and apoptosis were assessed by immunohistochemical Ki-67 staining and the TUNEL staining. There were significant differences in Ki-67 expression between the EGCG treatment group and control group, and the percentage of apoptotic cells in the EGCG treatment group was significantly greater than that in the control group. These results indicated that EGCG significantly inhibited cell proliferation by affecting the cell cycle progression and apoptosis *in vitro* and *in vivo*. These findings suggest that EGCG may have clinical applications as a novel approach to oral-cancer therapy.

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Key words: epigallocatechin-3-gallate, oral squamous cell carcinoma, cell proliferation, cell cycle, apoptosis, xenograft

Introduction

Oral cancer is one of the major head and neck malignant tumors. In terms of overall incidence, it is the sixth most common form of cancer and carries a high risk of recurrence (20-30%) (1). The annual worldwide incidence was 300,000 new cases in 2012, with two-thirds occurring in men (2). Although head and neck cancers generally form in the pharynx or larynx, ~50% of all cases involve the oral cavity (1). Such cancers most commonly involve the tongue and occur in the gingiva, buccal mucosa, floor of the mouth, palate, or lip. The significant risk factors contributing to the etiology of oral cancer are alcohol consumption and tobacco smoking (1). Histologically, >90% of oral cancers are diagnosed as oral squamous cell carcinoma (OSCC) (3). The 5-year survival rate of patients with OSCC has remained at ~50% for several decades in spite of advancements of treatment, including surgery, radiation, and chemotherapy (4). The poor survival rate is related to locoregional relapse and regional lymph node metastasis (5). Thus, OSCC is still a challenging disease to treat, and it is important to develop a novel antiproliferative treatment method for OSCC.

Green tea is a common beverage worldwide and has been studied regarding its usefulness for health (6,7).

An epidemiological study has revealed that green tea has preventive effects against cancer (8). The biological activity of green tea is attributed to its polyphenol components (constituting 30% of dry weight) (9). Polyphenols in green tea include many catechins-mainly epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC)-whereas EGCG is the most effective polyphenol (6). The use of EGCG has been shown to suppress cancer progression *in vitro* and in animal models: Not only the initiation but also progression or metastasis, in several cancer types such as lung, liver, breast, colorectal, prostate and skin cancer (9). Nonetheless, to the best of our knowledge, few studies have addressed the effect of EGCG on human OSCC cells, especially in an experimental animal model. In this study, we evaluated the influence of EGCG on a human OSCC cell line, HSC-3, then on an *in vivo* xenograft mouse model, by investigating cell proliferation and apoptosis. Finally, we discuss the therapeutic potential of EGCG for oral-cancer therapy.

Materials and methods

Reagents. EGCG was purchased from Sigma-Aldrich (cat. no. E4143), and cell titer 96® aqueous one solution cytotocity assay (an MTS assay kit) from Promega. Propidium iodide (PI) was acquired from Cayman Chemical (cat. no. 14289), whereas ribonuclease A from Sigma-Aldrich (cat. no. R6513). ApoScreen® Annexin V Apoptosis kit-FITC was bought from Southern Biotech Birmingham, and Amplite™ Fluorimetric Caspase-3/7 Activity kit from AAT Bioquest. The *In situ* Apoptosis Detection kit (TdT-mediated dUTP nick end labeling (TUNEL) assay) was purchased from Takara Bio, Inc., a rabbit anti-Ki-67 monoclonal antibody (cat. no. ab16667) from Abcam, and staurosporine and other chemicals from Wako Pure Chemical Industries, Ltd.

Cell culture conditions. The HSC-3 cell line (purchased from the Japanese Cancer Research Resources Bank, Tokyo, Japan) was used in this study. This cell line consists of primary tumor cells originating from a moderately differentiated squamous cell carcinoma (SCC) of the human tongue with lymph node metastasis (3). This cell line is one of the most commonly used for experimental study of OSCC with an allusion to their origin and biological behavior (3). The cells were cultured in the α-minimum essential medium (α-MEM; Invitrogen; Thermo Fisher Scientific, Inc.) with 10% of fetal calf serum (FCS; BioWest, Nuaillé, France). Penicillin (100 IU/ml) and streptomycin (100 mg/ml) (Invitrogen; Thermo Fisher Scientific, Inc.) were added to the medium. The cells were grown at 37˚C in a humidified atmosphere containing 5% of CO_2. The cells were subcultured every 3 days when confluence reached 80%.

The MTS assay. For this cytotoxity assay, $5x10^3$ cells were seeded in 96-well plates in α-MEM with 10% of FCS and cultured for 24 h. Then, the cells were treated with various concentrations of EGCG (0, 25, 50, 75 and 100 μM) in 100 μl of α-MEM with 1% of FCS for 24, 48 and 72 h. Cell viability was assessed by the MTS assay according to the manufacturer's instructions. Bioreduction of tetrazolium was measured as absorbance at 490 nm on a 96-well plate reader (SpectraMax M5; Molecular Devices), and the growth inhibition rate was calculated.

Cell cycle analysis. A total of 10^6 cells were seeded in a 10 cm dish containing α-MEM with 10% of FCS and were cultured for 24 h. Then, the cells were incubated with or without 50 μM EGCG in α-MEM with 5% of FCS for 24 h. The cells after treatment (0 and 24 h) were collected, washed with phosphate-buffered saline (PBS) twice, and fixed with 70% ethanol at -20˚C overnight. Then, the cells were centrifuged and washed with PBS twice, resuspended in 900 μl of PBS containing 0.25 mg/ml ribonuclease A, and incubated at 37˚C for 30 min, followed by addition of PBS with 100 μl of 500 μg/ml PI, and incubated at 4˚C for 15 min in the dark. Lastly, DNA contents were analyzed on a flow cytometer (BD FACSCanto II; BD Biosciences).

Annexin V-FITC analysis. The proportion (%) of apoptotic cells was determined according to the manufacturer's manual by means of the Annexin V Apoptosis kit-FITC and the flow cytometer. Namely, 10^6 cells were seeded in a 10 cm dish in α-MEM with 10% of FCS and cultured for 24 h. Next, the cells were incubated with or without 50 μM EGCG in α-MEM with 1% of FCS for 6 h. Five hundred nM staurosporine served as a positive control. The cells were collected, washed with PBS twice, and resuspended in cold Annexin binding buffer to a concentration of 10^6 cells/ml. Then, 10 μl of the Annexin V-FITC conjugate in a buffer was added to the cells and incubated for 15 min at 4˚C with protection from light. Finally, we added 10 μl of a PI solution in a buffer and analyzed the cells by flow cytometry. Annexin V-FITC$^+$/PI$^-$ cells are defined as early apoptotic cells, and Annexin V-FITC$^+$/PI$^+$ cells are defined as late apoptotic cells.

Measurement of caspase-3 and -7 activities. These activities were determined with the Amplite™ Fluorimetric Caspase-3/7 Activity kit. A total of $5x10^4$ cells per well were seeded in 96-well plates in α-MEM with 10% of FCS and cultured for 24 h. Then, the cells were incubated with or without 50 μM EGCG in α-MEM with 1% of FCS for 12 h. Five hundred nM staurosporine served as a positive control. The cells were rinsed once with ice-cold PBS and cultured in 100 μl of PBS. Subsequently, 1 μl of a caspase-3 and -7 inhibitor (Ac-DEVD-CHO, a synthetic tetrapeptide competitive inhibitor with a sequence Ac-Asp-Glu-Val-Asp-CHO, which contains the amino acid sequence of the PARP cleavage site) was added to selected samples as a negative control. The cells were lysed with 100 μl of the caspase-3/7 assay solution, and the plate was incubated for 2 h at room temperature with protection from light. Cleavage substrate fluorescence was measured on a 96-well plate reader at Excitation/Emission=350/450 nm (SpectraMax M5; Molecular Devices).

TUNEL staining in vitro. in vitro apoptosis was determined by the TUNEL assay using the *In situ* Apoptosis Detection kit (Takara Bio, Inc.). A total of $5x10^4$ cells per well were seeded in 8-well slide chambers (Watson Bio Laboratory) in α-MEM with 10% of FCS and were cultured for 24 h. After that, the cells were incubated with or without 50 μM EGCG in α-MEM with 1% of FCS for 24 h. Five hundred nM staurosporine served as a positive control. The cells were rinsed once with ice-cold PBS and fixed with 4% paraformaldehyde in PBS (pH 7.4). Thereafter, the endogenous peroxide activity was

eliminated with 0.3% H_2O_2 methanol solution for 30 min at room temperature. The cells were dipped in permeabilization buffer for 5 min at 4˚C. The cells were incubated with 50 μl of a labeling reaction mixture (consisting of TdT Enzyme 5 μl + Labeling Safe Buffer 45 μl) in a 37˚C humidified chamber for 90 min. Then, they were reacted with 50 μl of an anti-FITC antibody conjugated with horseradish peroxidase (not diluted, cat. no. MK503; Takara Bio, Inc.) for 30 min at 37˚C. Immunoreactivity was visualized by immersing the sections in 3,3'-diaminobenzidine (DAB; Dako, Agilent Technologies, Inc.) for 10 min at room temperature. Subsequently, the sections were counterstained with 3% methyl green for 1 min, dehydrated in 100% ethanol, which was replaced by Clear Plus (Falma Co., Ltd., Tokyo, Japan). Apoptotic cells were counted at magnification x400. The proportion of apoptotic cells was calculated by counting the TUNEL-positive cells and by dividing this number by the total number of tumor cells and then multiplying by 100%, in a minimum of seven microscopic fields under a light microscope.

The in vivo xenograft murine model. A total of 20 BALB/c nude (nu/nu) mice (5-week-old females; CLEA Japan, Inc.) were used in this study. The mice were kept in groups of three or four per cage and provided with food and water *ad libitum*. The animals were maintained under the following conditions: 12 h light/12 h dark cycle, 24˚C±2˚C, and 50±10% relative humidity (mean, range). A total of $5x10^6$ HSC-3 cells in 50 μl of α-MEM supplemented with 10% FCS were mixed with 50 μl of Matrigel (BD Biosciences) and implanted subcutaneously into the back of anesthetized mice. Tumor-bearing mice were subdivided into the EGCG group and control group. Two weeks after HSC-3 cells implantation, 75 mg/kg EGCG or saline was intraperitoneally injected via a syringe as described in previous reports (10,11). EGCG was administered twice a week for 4 weeks. The tumors were allowed to reach ~40 mm^3 in size. Body weight was recorded, and weight loss exceeding 20% was assumed to be a humane endpoint for euthanasia. Tumor diameters were measured with vernier calipers. Tumor volume was calculated by means of the following formula: (0.5 x length x $width^2$), as described by Yoshida *et al* (12). The mice were then euthanized, and the tumor was carefully removed along with the overlying skin and the surrounding tissue. The specimens were fixed with 4% paraformaldehyde and embedded in paraffin for histological examination. All the animal experiments were permitted by the Animal Ethics Committee of the University of Fukui (approval no. 27110) and followed the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA).

Immunohistochemistry. The tissue sections were deparaffinized in Clear Plus for 15 min and rehydrated in ethanol solutions of descending concentrations. For antigen retrieval, the sections were immersed in Tris-EDTA buffer (pH 9.0) and incubated at 95˚C for 30 min. The endogenous peroxide activity was eliminated with 0.3% H_2O_2 methanol solution for 30 min at room temperature. Normal horse serum (2.5%) for 30 min at room temperature was used to block nonspecific immunoreactions. The sections were incubated with a rabbit anti-Ki-67 monoclonal antibody (dilution 1:100) for 120 min at room temperature. The sections were then incubated with ImmPRESS (peroxidase) polymer anti-rabbit IgG reagent (cat. no. MP-7401; Vector Laboratories) for 30 min at room temperature. Immunoreactivity was visualized by immersing the sections in DAB. Subsequently, the sections were counterstained with hematoxylin for 30 sec at room temperature, dehydrated in ethanol solutions with ascending concentration, and finally incubated with Clear Plus at room temperature. The sections were rinsed three times in PBS between all the steps. The percentage of Ki-67-positive cells among all tumor cells was determined by cell counting in at least seven microscopic fields of vision (at magnification x400) per histological section.

TUNEL staining in vivo. The TUNEL assay was carried out to evaluate apoptosis using the *In situ* Apoptosis Detection kit (Takara Bio, Inc.). Briefly, the sections were deparaffinized in Clear Plus for 15 min, rehydrated in 100% ethanol for 15 min, and permeabilized with 10 μg/ml proteinase K (Invitrogen; Thermo Fisher Scientific, Inc.) for 10 min at room temperature. The endogenous peroxide activity was eliminated with 0.3% H_2O_2 methanol solution for 5 min at room temperature. The sections were incubated with 50 μl of a labeling reaction mixture (consisting of TdT Enzyme 5 μl + Labeling Safe Buffer 45 μl) in a 37˚C humidified chamber for 90 min. Then, they were reacted with 70 μl of the anti-FITC antibody conjugated with horseradish peroxidase (not diluted, cat. no. MK503; Takara Bio, Inc.) for 30 min at 37˚C. Immunoreactivity was visualized by immersing the sections in DAB for 10 min at room temperature. Next, the sections were counterstained with hematoxylin for 10 sec and dehydrated in 100% ethanol, which was replaced by Clear Plus. EXCEL Mount (Falma) served for mounting. The sections were rinsed three times in PBS between all the steps. The proportion of apoptotic cells was evaluated by counting the TUNEL-positive cells among all tumor cells, avoiding necrotic tumor areas, in a minimum of seven visual fields in each individual section under a light microscope (at magnification x400).

Statistical analysis. All statistical analyses were performed in the StatMate software (version 1.1; ATMS Co., Ltd.; for Macintosh). The measured values were presented as the mean with standard deviation (SD). Differences between groups were analyzed with unpaired t test (cell cycle, apoptosis cells *in vitro*, TUNEL staining *in vitro*, tumor volume, body weight, Ki-67 staining *in vivo* and TUNEL staining *in vivo*) or one-way ANOVA with Tukey-Kramer's multiple comparison post-hoc test (cell viability and caspase-3/7 activity). $P<0.05$ was considered to indicate a statistically significant difference.

Results

EGCG suppresses HSC-3 cell proliferation in vitro. To examine the effect of EGCG on OSCC cells, HSC-3 cells were cultured with different concentration of EGCG for various periods. Based on the cell viability, the inhibition rate was calculated by means of the MTS assay. EGCG significantly inhibited cell viability in a dose- and time-dependent manner (Fig. 1). The IC_{50} value at 24, 48 and 72 h were >100, 43.2 and 39.3 μM, respectively. There was no significant difference in inhibitory

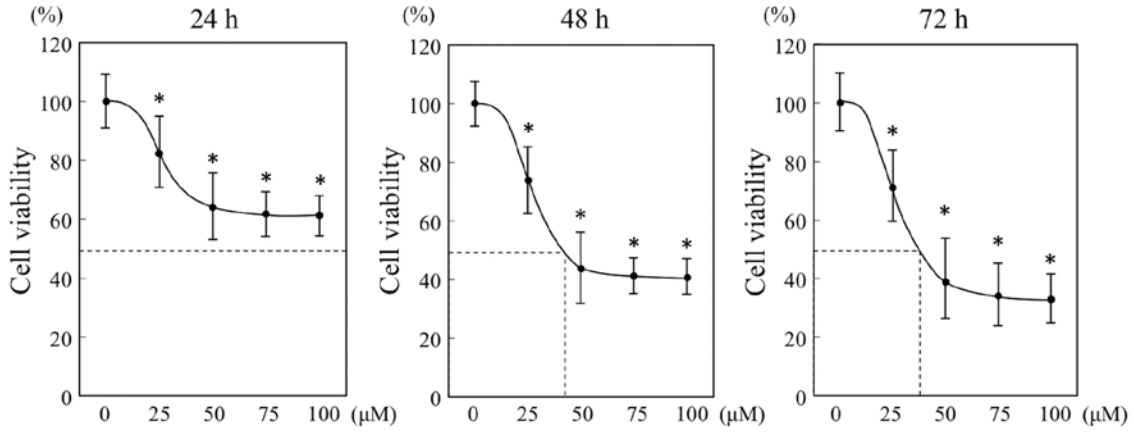

Figure 1. EGCG inhibits HSC-3 cells growth. HSC-3 cells were treated with various doses of EGCG (0, 25, 50, 75 and 100 μM) for 24, 48 and 72 h. The data represent the mean ± SD from three independent experiments; *P<0.05 vs. control. The IC_{50} value at 24, 48 and 72 h were >100, 43.2 and 39.3 μM, respectively. EGCG, epigallocatechin-3-gallate.

effect at concentrations above 50 μM. Thus, we used 50 μM of EGCG for the following experiments.

EGCG arrests HSC-3 cells in the G1 phase in vitro. To identify the mechanism by which EGCG influences cell viability, we performed cell cycle analysis. We examined the DNA content of HSC-3 cells treated with or without WGCG treatment at 0 and 24 h. The flow-cytometric analysis revealed a significant increase in the percentage of G1 phase cells as compared to control cells (64.3±4.5 vs. 46.2±4.7%), and a significant decrease in the percentage of G2/M phase cells as compared with control cells (11.6±5.1 vs. 26.0±5.5%) at 24 h (Fig. 2A and B). These results showed that EGCG can arrest HSC-3 cells at the G1 checkpoint of the cell cycle.

EGCG induces apoptosis of HSC-3 cells in vitro. To further investigate the mechanism, we have evaluated the effects of EGCG on three stages of apoptosis: The early stage by Annexin V assay which estimates the expression of phosphatidylserine on the outer leaflet of the cell membrane, the middle stage by evaluation of the activities of caspase-3 and -7 (13,14), and the late stage by TUNEL assay which detects DNA strand breaks. The cells were incubated with or without EGCG for 6 h and then analyzed by flow cytometry with Annexin V to determine the apoptotic rate. The treatment with EGCG significantly increased the percentage of apoptotic cells as compared to control cells (early apoptotic cells: 2.6±0.4 vs. 0.6±0.2% and late apoptotic cells: 21.0±1.2 vs. 3.8±0.6%) (Fig. 3A and B). We also assessed the effects of EGCG on the activities of caspase-3 and -7. The caspase-3 and -7 activities significantly increased after 12 h of treatment with EGCG as compared to control cells (2,861.4±580.7 RFU vs. 884.6±76.6 RFU) (Fig. 3C). The caspase-3 and -7 inhibitor (Ac-DEVD-CHO) was used in this study, and it significantly suppressed these activities. We also investigated the effect of EGCG on apoptosis *in vitro* by the TUNEL assay. The percentage of apoptotic cells of EGCG treatment for 24 h was significantly greater than that in the control cell group (2.8±1.1 vs. 0.3±0.5%) (Fig. 3D and E). These results suggested that EGCG induces apoptosis in HSC-3 cells.

EGCG suppresses tumor growth of HSC-3 cells in vivo. To confirm the *in vitro* findings above, we examined the effects of EGCG on *in vivo* tumor growth. HSC-3 cells were implanted subcutaneously into the back of nude mice, with monitoring for 6 weeks. Two weeks after HSC-3 cells implantation, 75 mg/kg EGCG or saline was intraperitoneally administered twice a week for 4 weeks. A significant difference was observed from 1 week after the administration of EGCG. The administration of EGCG for 4 weeks resulted in a 45.2% reduction in tumor volume as compared with control animals (46.7±17.8 vs 103.4±12.4 mm^3) (Fig. 4A-C). The weight of the mice did not decrease significantly, and the average weight of the EGCG treatment group was 23.3±1.5 g, almost equal to that of the control group (24.2±1.2 g) (Fig. 4D). In agreement with *in vitro* data, EGCG significantly inhibited tumor growth in our xenograft model.

EGCG suppresses division of HSC-3 cells in vivo. Ki-67 is a 395 kDa nuclear antigen whose expression is confined to the late G1, S, M and G2 phases, and its expression is strictly associated with cell proliferation (15). We also evaluated the expression of Ki-67 in tumor cells *in vivo*. There were significant differences in mean Ki-67 expression between the EGCG treatment and the control group (8.8±3.2 vs. 5.0±2.4%) (Fig. 5A and B). The results indicated an inhibitory effect of EGCG on cell proliferation in the xenograft tumors.

EGCG induces apoptosis of HSC-3 cells in vivo. We investigated the effect of EGCG on apoptosis *in vivo* by the TUNEL assay. The percentage of apoptotic cells in the EGCG treatment group was significantly greater than that in the control group (10.6±4.2 to 4.4±2.3%) (Fig. 6A and B). Thus, the EGCG treatment can significantly induce apoptosis in the xenograft tumors.

Discussion

Oral cancer is one of the most common malignant tumors in the head and neck region (5), and >90% of oral cancers are OSCC (3). Patients with oral cancer have a high mortality rate because of local invasion and distant metastasis (5). The 5-year survival rate of patients with early-stage disease is ~80%, and this rate of late-stage disease is only ~20% (16). Because the

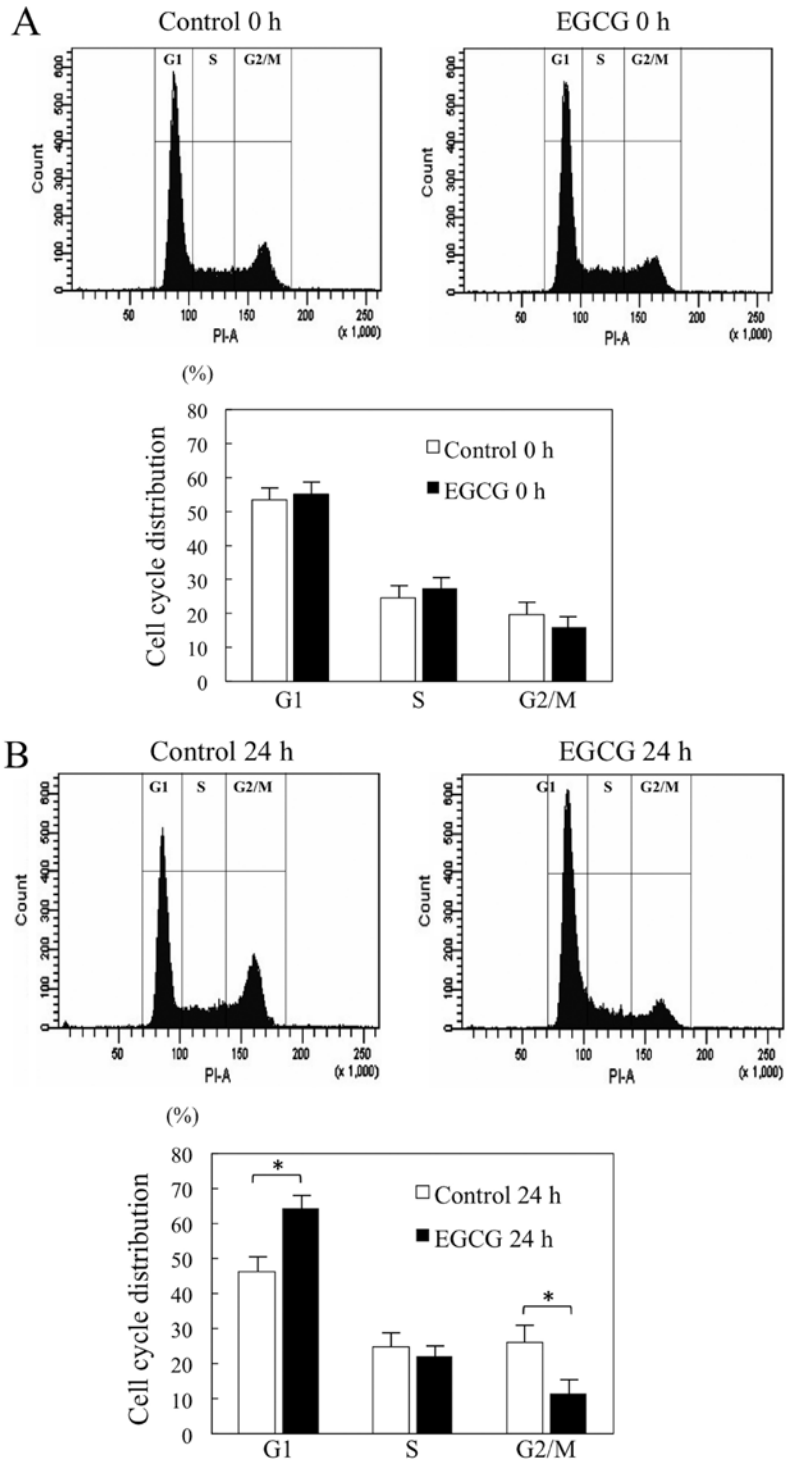

Figure 2. EGCG arrests HSC-3 cells in the G1 phase. Cells were incubated with or without 50 μM EGCG for (A) 0 h and (B) 24 h. The first peak showed cells in the G1 phase, the second peak indicated cells in the G2/M transition and the area between the two peaks showed an S phase. Representative results of three independent experiments are shown. Graphical representation of the percentage of cells in the G1, G2/M, and S phases of the cell cycle in control and EGCG-treated cells. The data represent the mean ± SD from three independent experiments; *P<0.05 vs. control. There were significant differences in G1 phase and G2/M phase, while there was no significant difference in S phase between control and EGCG-treated cells at 24 h. EGCG, epigallocatechin-3-gallate.

most advanced oral cancer is impossible to heal, blocking the process of carcinogenesis is an important strategy for cancer management.

Green tea has been a popular beverage for many centuries (7). The main polyphenols in green tea are catechins, and the four main catechins are EGCG (constitutes 59% of all catechins), EGC (19%), ECG (13.6%), and EC (6.4%) (7). Particularly, its major constituent, EGCG, has been demonstrated to act on several key components of intracellular signaling pathways associated with cell proliferation, differentiation, apoptosis, inflammation, angiogenesis, and metastasis; however, these molecular mechanisms are not completely characterized, and many features have not been elucidated (17). We herein examined the possibility that

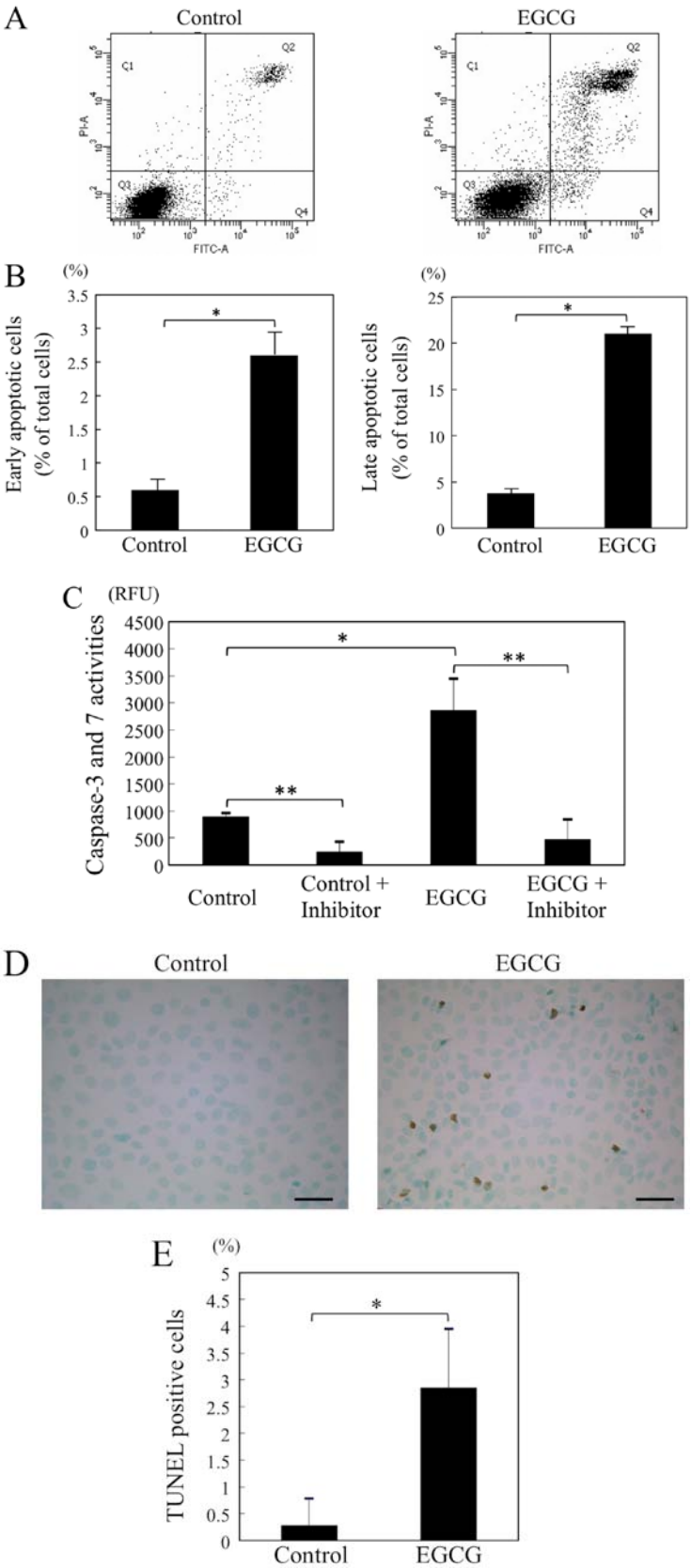

Figure 3. EGCG induces apoptosis in HSC-3 cells. (A) Cells were incubated with or without 50 μM EGCG for 6 h. Annexin V-FITC$^+$/PI$^-$ cells are defined as early apoptotic cells, and Annexin V-FITC$^+$/PI$^+$ cells are defined as late apoptotic cells. Representative cytograms from three independent experiments are shown. (B) Graphical representation of the percentage of early and late apoptotic cells in control and EGCG-treated cells. The data represents the mean ± SD from three independent experiments; *P<0.05 vs. control. (C) Cells were incubated with or without 50 μM EGCG for 12 h. Caspase-3 and -7 activities were measured as described in Materials and methods. Ac-DEVD-CHO served as a caspase-3 and -7 inhibitor. The data represent the mean ± SD from three independent experiments; *P<0.05, compared to the control. **P<0.05, compared to the caspase-3 and -7 inhibitor. RFU, Relative fluorescence units. (D) Cells were incubated with or without 50 μM EGCG for 24 h. An *in vitro* TUNEL assay was carried out to assess the rate of apoptotic cell death. A representative image of the TUNEL assay of control and EGCG-treated cells (magnification, x400, scale bar, 50 μm). (E) Graphical representation of the results in control and EGCG-treated cells. The data represent the mean ± SD from three independent experiments; *P<0.05 vs. control. EGCG, epigallocatechin-3-gallate.

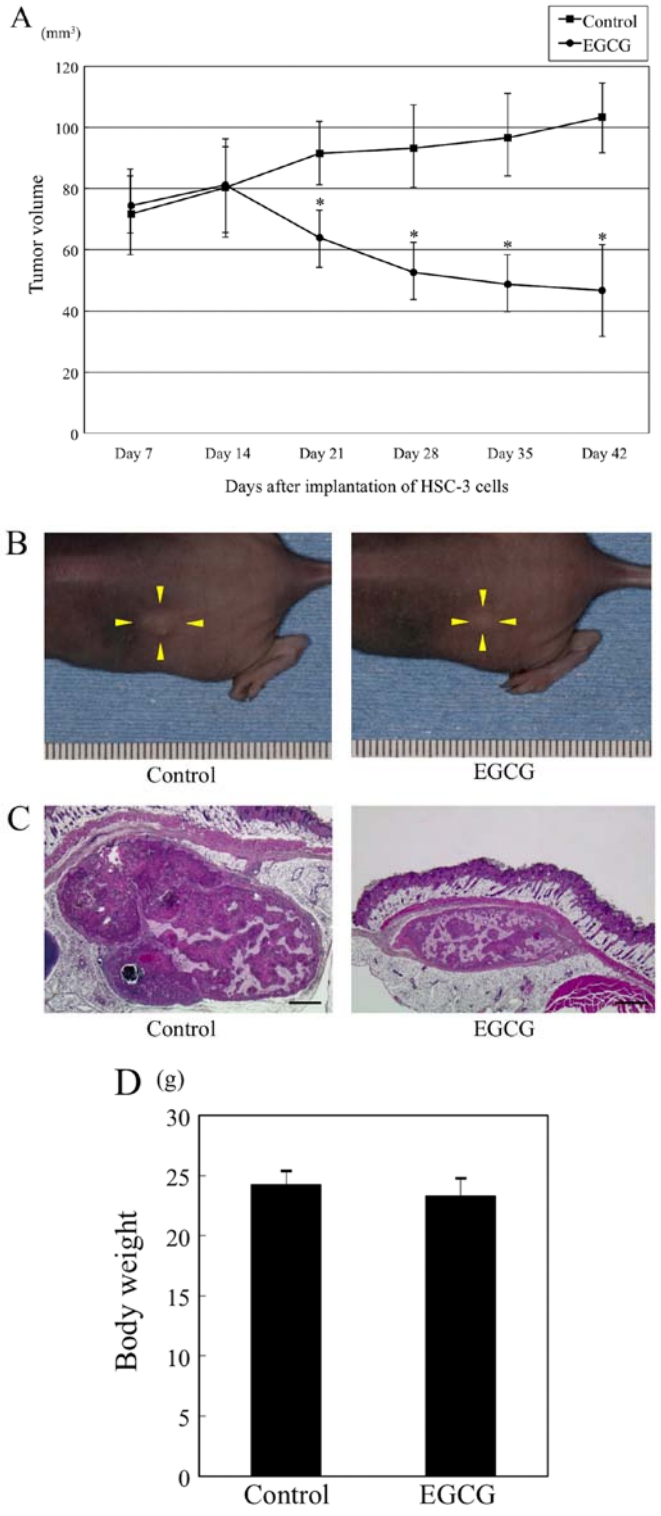

Figure 4. EGCG inhibits tumor formation in a xenograft model. (A) Graphical representation of tumor volume in control and EGCG-treated mice. Two weeks after the HSC-3 cells implantation, 75 mg/kg EGCG or saline was intraperitoneally administered twice a week for 4 weeks. The data represent the mean ± SD; *P<0.05 vs. control. (B) Representative images of implanted HSC-3 tumors of control and EGCG-treated mice at 42 days after implantation. (C) Histological evaluation of tumors in control and EGCG-treated mice at 42 days after implantation. (H&E staining, magnification x200, scale bar, 500 μm). (D) Graphical representation of the body weight of control and EGCG-treated mice at 42 days after implantation.

EGCG may have a therapeutic potential for human oral cancer.

First, we examined the effectiveness of EGCG against the cultured OSCC cell line, HSC-3. After exposure to EGCG, HSC-3 cells manifested suppression of cellular proliferation and were arrested in the G1 phase of the cell cycle. Elattar and Virji have demonstrated a significant inhibitory effect of EGCG on the growth and proliferation of OSCC cells (SCC-25), where high doses of EGCG exert an inhibitory effect on DNA synthesis (18). Masuda *et al* have reported that EGCG inhibits cell growth and causes G1 arrest of the cell cycle in two cell lines derived from human head and neck squamous cell carcinoma (HNSCC): YCU-H891 and YCU-N861 (19). In the present study, these reported effects of EGCG on OSCC and HNSCC could also be observed in HSC-3 cells *in vitro*. Apoptosis is a form of programmed cell death and plays an important role in the regulation of cell homeostasis of eukaryotes (20). Apoptosis can proceed via the mitochondria-mediated (intrinsic) pathway (including Bax, Bcl-2, and Bcl-X_L proteins) and a death receptor-mediated (extrinsic) pathway (including Fas/CD95 and FADD) (20), and can be induced by agents such as drugs used for chemotherapy (21). Masuda *et al* reported that EGCG induces apoptosis in two cell lines of human HNSCC. Cell death in both cell lines was found to be related to downregulation of the antiapoptotic Bcl-X_L and Bcl-2 proteins and an increase in the amount of the proapoptotic Bax protein (19). Lin *et al* have demonstrated dose- and time-dependent apoptosis of EGCG-treated HNSCC cells (SAS and Cal-27) (22). Apoptosis is induced through the expression of Fas/CD95. In line with increased Fas/CD95 expression, EGCG inhibits STAT3 phosphorylation, translocation to the nucleus, and leads to downregulation of the target gene products of STAT3, such as Bcl-2, Mcl-1, VEGF and cyclin D1 (22). Thus, EGCG can induce apoptosis through effects on the mitochondria-mediated and the death receptor-mediated pathway. Caspases are a family of intracellular cysteine proteases and are known to perform an important function in the initiation and execution of apoptosis (23). The mitochondria-mediated and death receptor-mediated pathways converge at the level of caspase-3 activation (20), whereas caspase-3 and -7 serve as effectors of apoptotic cells (13,14). We evaluated the effect of EGCG on caspase-3 and -7 activities, and the results suggested that the proapoptotic effect of EGCG in HSC-3 cells is mediated by activation of those caspases. We may need to verify the detailed mechanism of EGCG's action on cell cycle arrest and apoptosis as well as its effect on the molecular pathway. Nevertheless, our results confirmed that EGCG significantly inhibits HSC-3 cell proliferation by at least affecting the cell cycle and apoptosis *in vitro*. In this study, we did not examine the toxicity of EGCG on normal cells. Previous reports have shown that EGCG had no effect on normal cells. Yamamoto *et al* reported that a high dose of EGCG (~200 μM) caused reactive oxygen species production and apoptosis only in oral SCC, but not in normal epithelial cells *in vitro* (24). Chen *et al* reported that tea polyphenols potently induced apoptotic cell death and cell cycle arrest in tumor cells without affecting the normal cell counterparts (25). Further research will be required to evaluate the differential effect of EGCG on cancer cells and normal cells.

To develop effective therapeutic strategies, reliable experimental models are important. An *in vivo* xenograft mouse model is one of the reliable experimental models for the development of more effective treatments with evaluation of cytotoxic effects (26). Second, we examined the significance

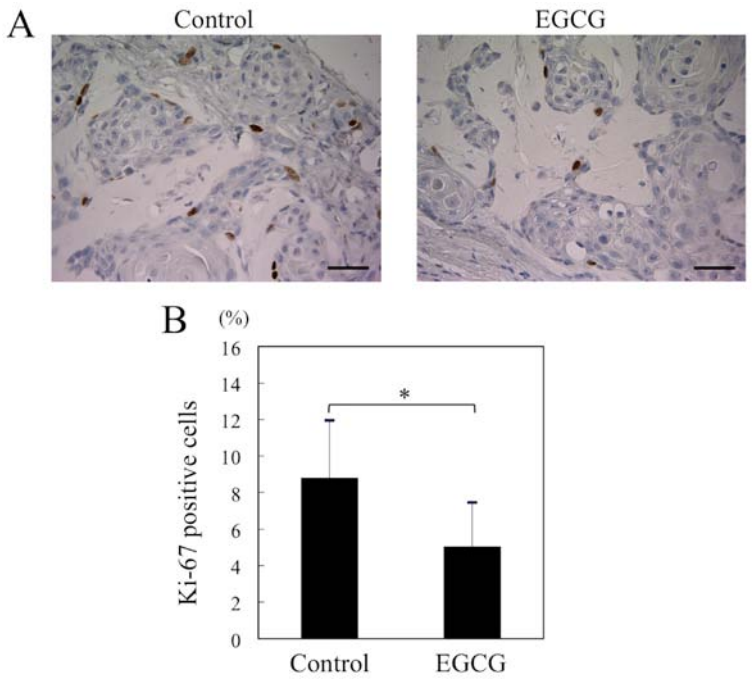

Figure 5. EGCG suppresses Ki-67 expression *in vivo*. (A) Immunohistological evaluation of Ki-67 expression in control and EGCG-treated mice (magnification, x400, scale bar, 50 μm). (B) Graphical representation of Ki-67 expression in control and EGCG-treated mice. The data represent the mean ± SD; *P<0.05 vs. control. EGCG, epigallocatechin-3-gallate.

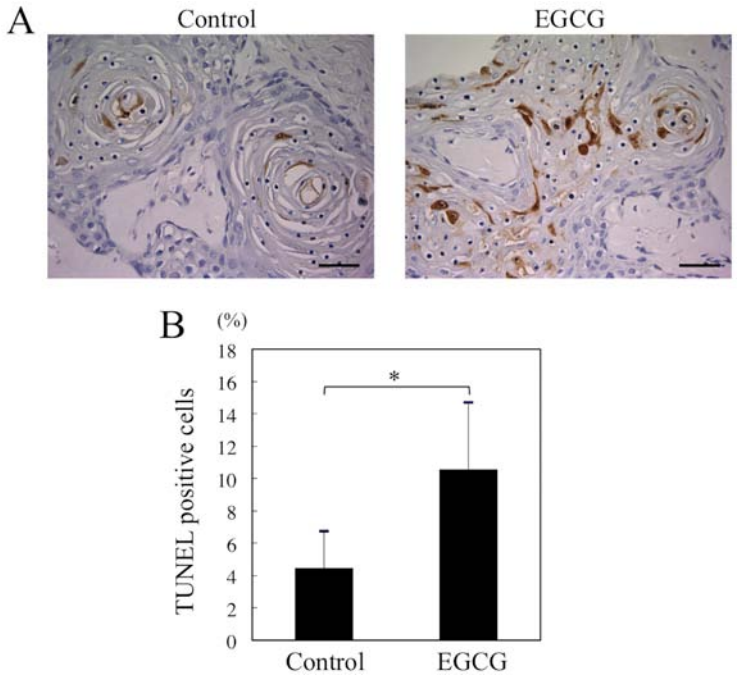

Figure 6. EGCG induces apoptosis *in vivo*. (A) A representative image of the TUNEL assay in control and EGCG-treated mice (magnification, x400, scale bar, 50 μm). (B) Graphical representation of the TUNEL assay results on control and EGCG-treated mice. The data denote the mean ± SD; *P<0.05 vs. control.

of EGCG effects on tumor growth by means of the xenograft mouse model. Nude mice were intraperitoneally injected with or without 75 mg/kg EGCG. Our *in vivo* experiments indicated that, compared to the control group, there was substantially diminished tumor growth in the EGCG-treated group, with inhibition of cell division (Ki-67) and induction of apoptosis (TUNEL assay). Many *in vitro* assays involving an OSCC cell line have shown the effects of EGCG, but few researchers have reported *in vivo* xenograft models (Table I) (27-29). To our knowledge, this is the first report that documents antiproliferative and proapoptotic effects of EGCG on oral cancer cells *in vivo*. Although there was a difference in the cancer-suppressive effects of EGCG between the *in vitro* and *in vivo* experiments, this difference might be attributed to the OSCC cell environment and concentration of EGCG.

Table I. Studies investing the effects of tea contents on human oral malignancy in xenograft mouse model.

		Administration of tea extract				
Author (year)	OSCC cell	Content	Rote	Dose and duration	Results	(Refs.)
Chen *et al* (2011)	SCC-9 (human tongue SCC)	EGCG	Oral administration	10-20 mg/kg, every day for 44 days	Inhibition of the tumor growth	(27)
Chang *et al* (2012)	SCC-4 (human tongue SCC)	Black tea extract	Oral administration	25, 50 mg/kg, every day for 45 days	Inhibition of the tumor growth	(28)
Hwang *et al* (2013)	YD-10B (human oral SCC)	EGCG	Intra-peritoneal injection	20 mg/kg, every other day for 28 days	Inhibition of the tumor growth. Inhibition the phosphorylation of Src, CTTN, FAK proteins. Inhibition of the expression of MT1-MMP, MMP-2, MMP-9.	(29)
Present study (2019)	HSC-3 (human oral SCC)	EGCG	Intra-peritoneal injection	75 mg/kg, twice a week for 28 days	Inhibition of the tumor growth. Increase in apoptotic index, and decrease in proliferation index.	

CTTN, cortactin; EGCG, epigallocatechin-3-gallate; FAK, focal adhesion kinase; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-matrix metalloproteinase; OSCC, oral squamous cell carcinoma; Src, sarcoma; SCC, squamous cell carcinoma.

In animal experimental models, weight loss is the marker of adverse effects or toxicity of a pharmacological agent. In our study, the weight in the EGCG-treated group of mice was almost equal to that of the control group. Thus, our study also revealed that EGCG exerted a milder adverse effects, while it was still able to induce apoptosis and cell cycle arrest in OSCC cells. Due to its pharmacological properties and reduced adverse effects, EGCG may be a promising agent and a novel approach to oral-cancer therapy.

In this study, a time and dose discrepancy in the cancer-suppressive effects of EGCG was observed between the *in vitro* and *in vivo* experiments. We have used a range of EGCG concentrations from 25 to 100 μM for up to 3 days in the *in vitro* experiment, whereas 75 mg/kg EGCG was intraperitoneally administered twice a week for up to 28 days in the *in vivo* experiment. Generally, the concentration of EGCG used in cell culture experiments (20-100 μM) is much higher than the plasma and tissue concentrations observed in the cancer-prevention studies conducted in mice (usually <0.5 μM) (30,31). Although the *in vivo* concentration in this study was low, the prolonged exposure might have produced significant effects. This seems consistent with our observation *in vitro* that treatment of cancer cells with EGCG for a prolonged period (3 days) increases the extent of inhibition of cell viability compared to treatment for 1 or 2 days. The concentration and time of onset of effects may be clinically relevant. There is a possibility that the difference in the environment of the tumor cells might also have played a role. The difficulty in studying the biological activities of EGCG lies with correlating the *in vitro* biological effects with the proposed mechanisms based on the *in vivo* study results (31). Thus, the inhibitory activities of EGCG against carcinogenesis should be demonstrated both in *in vitro* and *in vivo* experiments.

It may also not be obvious whether the information obtained from cell lines with high EGCG concentrations can be extrapolated to cancer prevention in humans. However, the anticancer effect of EGCG has been demonstrated in epidemiological studies and in clinical trials (1,9,32). One epidemiological study has shown a protective effect of green tea against malignant tumors. In a study of 8552 Japanese adults, daily drinking >10 cups of green tea per day decreased the risk and delayed the onset of cancers compared to those who drank less than 3 cups per day (8). One clinical trial showed that green tea extract (2,000-2,500 mg/day) administration to smokers for 4 weeks reduces DNA damage in oral keratinocytes. In addition, cell growth is inhibited, the percentage of cells in the S phase decreases, cells accumulate in the G1 phase, and apoptotic markers are upregulated. Therefore, regular consumption of green tea or administration of green tea extracts could be beneficial for the prevention of oral cancer in humans.

In conclusion, we demonstrated that EGCG induces cell cycle arrest and apoptosis in human OSCC cells, resulting in antiproliferative effects *in vitro* and *in vivo*: In a mouse model, significant growth inhibition of the OSCC tumor was observed in EGCG-treated mice without a loss of body weight. Thus, we believe that EGCG is a potential anticancer agent for OSCC therapy. The mechanisms underlying the anticancer effects of EGCG seem to be complex. Further research, especially molecular and clinical, is needed to elucidate the usefulness of EGCG for oral-cancer therapy.

Acknowledgements

Not applicable.

Funding

This study was supported by a Grant-in-Aid for Scientific Research (grant nos. JP19791515, JP22890074, JP2472196

and JP15K11240) from the Japan Society for the Promotion of Science, Japan.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HYoshim made substantial contributions to conception and design, and acquisition of data and drafting the manuscript. HYoshid made substantial contributions to conception and design and acquisition of data. SM, TR and KO performed the experiments. MO and SY analyzed the data. TK, MK and KS wrote the manuscript and also made substantial contributions to conception of data, analysis of data and critically revising the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were permitted by the Animal Ethics Committee of the University of Fukui (approval no. 27110).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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