Effect of resveratrol on cancer progression through the *REG III* expression pathway in head and neck cancer cells

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Abstract. Identification of reliable markers of chemo- and radiosensitivity and the key molecules that enhance the susceptibility of head and neck squamous cell carcinoma (HNSCC) to anticancer treatments is highly desirable. Previously, we have reported that *regenerating gene (REG) III* expression was such a marker associated with an improved survival rate for HNSCC patients. In the present study, we investigated the stimulators for induction of *REG III* expression using *REG III* promoter assay in HNSCC cells transfected with *REG III* promoter vector. We tested inflammatory cytokines, growth factors, polyphenols, PPARγ activator of thiazolidinediones, and histone deacetylase inhibitors, and found that 3,4',5-trihydroxy-*trans*-stilbene (resveratrol) significantly increased the *REG III* promoter activity and the mRNA levels of *REG III* in HNSCC cells. Moreover, we demonstrated the effect of resveratrol on cancer cell progression, such as cell proliferation, chemo- and radiosensitivity and cancer invasion of HNSCC cells. Resveratrol significantly inhibited cell growth, enhanced chemo- and radiosensitivity, and blocked cancer invasion of HNSCC cells. These data suggested that resveratrol could inhibit cancer progression through the *REG III* expression pathway in HNSCC cells.

Introduction

Worldwide, >600,000 people are newly diagnosed with head and neck cancer each year (1-3), and head and neck squamous cell carcinoma (HNSCC) is one of the 10 most common malignancies in the world and also known for clinical progression and poor prognosis. The cancer-related death is mainly caused by metastasis of tumors to regional lymph nodes and distant organs. Many patients are diagnosed in advanced stages, in which standard treatment is a combination of platinum-based chemotherapy, radiation, and/or surgery (3-6). As primary treatments for advanced HNSCC, recently there have been advances in definitive chemoradiotherapy (CRT) instead of extended surgery. Despite the recent progress of the treatment for improving locoregional control in HNSCC patients, that for recurrence and metastatic control remains insufficient (7,8), indicating that there is a continued need for improved treatment strategies. As an obvious risk factor of HNSCC, continuous irritation by tobacco and alcohol abuse is well-known (3,7,9). Furthermore, recent studies have shown that HNSCC is associated with human papillomavirus (HPV) infection, especially at oropharyngeal region (10-12). In contrast, there is no reliable marker for the other sites of HNSCC. Therefore, the key molecules that enhance chemo- and radiosensitivity in HNSCC and identification of reliable markers for predicting the recurrence and metastasis would be desirable to improve the prognosis of HNSCC patients.

It has been reported that inflammation is an important phase in tumor progression, and many cancers originate from inflammation. In connection with digestive organs, many studies have demonstrated that the human *regenerating gene* (*REG*) expression is observed in chronic inflammation and in tumors (13-20). *Reg* was first identified in regenerating pancreatic islets in 1988 (21). Reg family belongs to the lectin superfamily and encodes five small, secreted proteins (13,22-25). Reg family proteins are classified into four subfamilies: type I, II, III, and IV; the human *REG* family consists of five members: *REG Iα, REG Iβ, REG III, hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein* (*HIP/PAP*) and *REG IV* (13,20,22,26-29). REG family protein has been shown to play roles, not only in normal tissue regeneration (30,31), but also in the development of various malignancies (32-43). Indeed, *REG* expression has been reported to be associated with progression of cancers such as esophageal, gastric, lung, and colorectal cancer (32-39,43). Considering that oral and pharyngeal cavities are often exposed to chronic inflammatory factors such as tobacco, alcohol and mechanical stimuli, it is possible that *REG* expression is associated with HNSCC. Recently we reported that *REG III* expression was associated with prognosis of HNSCC patients (44). In addition, we demonstrated that *REG III*

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regulated cell proliferation and chemo- and radiosensitivity in HNSCC *in vitro*. These results suggested that enhancement of *REG III* expression increased chemo- and radiosensitivity, and improved prognosis of HNSCC patients. In the present study, we investigated the stimulator for the enhancement of *REG III* expression in HNSCC cells. Furthermore, we first demonstrate that the stimulator can effect proliferation, invasion, and chemo- and radiosensitivity in HNSCC cells.

Materials and methods

Cell culture and reagents. Human HNSCC cells, FaDu and HSC-4, were employed in this study. FaDu cells were provided by the American Type Culture Collection (Manassas, VA, USA). HSC-4 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 100 U/ml penicillin G and 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Gibco™ Antibiotic-Antimycotic; Gibco). The cells were maintained in a 5% CO_2/95% air, humidified atmosphere at 37˚C. Interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) were purchased from Roche Applied Science (Indianapolis, IN, USA). IL-1β, IL-8, IL-11, IL-13, IL-17, IL-22, hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), cardiotrophin, oncostatin M, interferon (IFN)-β, quercetin, 3,4',5-trihydroxy-*trans*-stilbene (resveratrol), fisetin, genistein, chlorogenic acid, estradiol, bisphenol A, sodium butyrate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dexamethasone (Dx) and o-dianisidine were from MP Biomedicals (Santa Ana, CA, USA). Daidzein was purchased from Fujicco Co., Ltd. (Kobe, Japan), epigallocatechin gallate from Enzo Life Sciences, Inc. (Framingdale, NY, USA), curcumin from Nacalai Tesque, Inc. (Tokyo Japan), ciglitazone and troglitazone from Cayman Chemical Co. (Ann Arbor, MI, USA), trichostatin A from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and phorbol 12-myristate 13-acetate from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of stable transformants with REG III promoter vector. The reporter constructs were prepared by inserting the 5'-flanking regions of human *REG III* (-1,985 to +87 of *REG III* gene) (28) upstream of a luciferase reporter gene in pGL4.17 vector (luc2/Neo; Promega Corp., Madison, WI, USA). The *REG III* promoter vector was introduced into FaDu cells by electroporation using Gene Pulser Xcell™ (Bio-Rad, Hercules, CA, USA) (44), after which the stable transformants were selected in DMEM supplemented with 10% FBS and 500 μg/ml Geneticin® (Invitrogen) for 3 weeks. Introduction of the *REG III* promoter/luciferase construct in the Geneticin®-resistant cells was confirmed by PCR.

Promoter assays. The *REG III* promoter vector was introduced into FaDu cells as described above. The stable transformants were seeded in 24-well plates at an initial density of $1x10^5$ cells/well. After a 24-h incubation, the cells were treated with 20 ng/ml of IL-1β, 20 ng/ml of IL-6, 10 nM of IL-8, 100 ng/ml of IL-11, 100 ng/ml of IL-13, 1 μg/ml of IL-17, 20 ng/ml of IL-22, 50 ng/ml of HGF, 10 nM of bFGF, 10 nM of EGF, 20 ng/ml of TNF-α, 20 ng/ml of cardiotrophin, 20 ng/ml of oncostatin M, 100 nM of Dx, 50 ng/ml of IFN-β, 50 μM of quercetin, 20 μM of resveratrol, 20 μM of fisetin, 50 μM of genistein, 60 μM of chlorogenic acid, 200 μM of o-dianisidine, 10 nM of okadaic acid, 10 μM of daidzein, 100 nM of epigallocatechin gallate, 1 μM of estradiol, 10 μM of bisphenol A, 12.5 μM of curcumin, 50 μM of ciglitazone, 30 μM of troglitazone, 1 μM of trichostatin A, 1 mM of sodium butyrate, or 50 nM of phorbol 12-myristate 13-acetate. After a 24-h treatment, the medium was aspirated from each well and washed with PBS, and the cells were harvested with 100 μl of lysis buffer (0.1 M potassium phosphate, pH 8.8, 0.2% Triton X-100). The cells were processed for luciferase assay using a Pica Gene luminescence kit (TOYO B-Net Co., Ltd., Tokyo, Japan) as described (45-50).

Quantitative real-time RT-PCR. Total RNA was isolated using RNeasy Protect® Cell Mini Kit (Qiagen, Hiden, Germany) from FaDu cells. cDNA was reverse transcribed from 0.5-2 μg samples of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) as described (44,45,47-51). cDNA was subjected to PCR with the following primers, synthesized and prepared by Nihon Gene Research Laboratories, Inc. (NGRL; Sendai, Japan): β-actin (NM_001101) sense, 5'-GCGAGAAGATGACCCAGA-3' and antisense, 5'-CAGAGGCGTACAGGGATA-3'; *REG III* (AB161037) sense 5'-GAATATTCTCCCCAAACTG-3' and antisense, 5'-GAGAAAAGCCTGAAATGAAG-3'.

Real-time PCR was performed using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Boston, MA, USA) and Thermal Cycler Dice Real-Time System (Takara Bio, Inc., Otsu, Japan) as described (43-45,47-51). PCR was performed with an initial step of 3 min at 95˚C followed by 40 cycles of 3 sec at 95˚C, 20 sec at 60˚C. The level of target mRNA was normalized to the mRNA level of *β-actin* as an internal standard.

Cell proliferation assay. Cell proliferation activity was assessed by using a Cell Counting Kit-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) cleavage; Dojindo Laboratories, Kumamoto, Japan] as described (43,44,47,50,52). Cells were plated in 96-well plates and incubated for 24 h. Initial density of FaDu and HSC-4 cells was $3x10^3$ cells/well. After 24 h, 30 μM of resveratrol was added to each well and incubated for 24, 48 and 72 h, while dimethylsulfoxide (DMSO; Sigma-Aldrich) was added as a control. At 24 h of incubation, medium was changed and resveratrol was removed from each well. After addition of 10 μl of WST-8 solution, the cells were incubated for another 2 h. The absorbance of each well at 450 nm (reference wavelength at 620 nm) was read by using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA). Each measurement was repeated at least four times on each cell line.

Chemo- and radiotherapy for cultured cells. Cells were exposed to 0, 5 and 10 Gy radiation using a MBR-1520R (Hitachi, Ltd., Tokyo, Japan) operating at 150 kV and 20 mA as

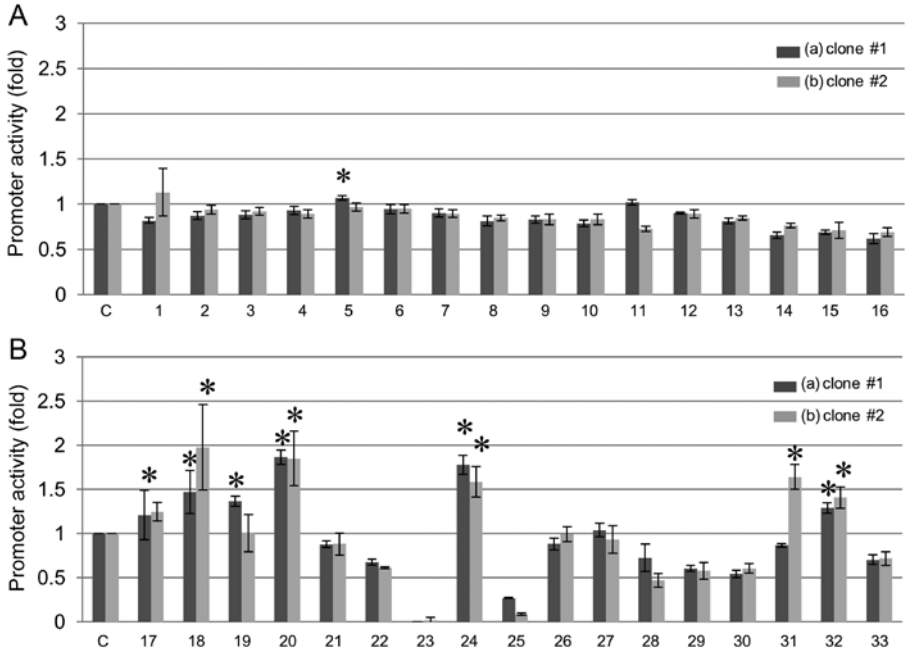

Figure 1. Screening of activator(s) for human *REG III* transcription. Human *REG III* promoter (-1,985 to +87 of *REG III* gene) was introduced into FaDu human HNSCC cells and stable transformants were selected (clone #1 and #2). (A) Effects of cytokines and growth factors on activation of *REG III* gene promoter of (a) clone #1, (b) clone #2 in FaDu cells. Cells were transfected with the *REG III* gene reporter plasmids and treated as follows: C, no addition as a control; 1, IL-1β (20 ng/ml); 2, IL-6 (20 ng/ml); 3, IL-8 (10 nM); 4, IL-11 (100 ng/ml); 5, IL-13 (100 ng/ml); 6, IL-17 (1 μg/ml); 7, IL-22 (20 ng/ml); 8, HGF (50 ng/ml); 9, bFGF (10 nM); 10, EGF (10 nM); 11, TNF-α (20 ng/ml); 12, cardiotrophin (20 ng/ml); 13, oncostatin M (20 ng/ml); 14, Dx (100 nM); 15, IL-6 + Dx; 16, IFN-β (50 ng/ml). The relative promoter activity was calculated by dividing the promoter activity of unstimulated cells (column 1). Data are expressed as means ± SE for each group. *P<0.05. (B) Effects of polyphenols, epigallocatechin gallate, PPARγ activator of thiazolidinediones, and histone deacetylase inhibitor on activation of *REG III* gene promoter of (a) clone #1, (b) clone #2 in FaDu cells. Cells were transfected with the *REG III* gene reporter plasmids and treated as follows: C, no addition as a control; 17, quercetin (50 μM); 18, resveratrol (20 μM); 19, fisetin (20 μM); 20, genistein (50 μM); 21, chlorogenic acid (60 μM); 22, o-dianisidine (200 μM); 23, okadaic acid (10 nM); 24, daidzein (10 μM); 25, epigallocatechin gallate (100 nM); 26, estradiol (1 μM); 27, bisphenol A (10 μM); 28, curcumin (12.5 μM); 29, ciglitazone (50 μM); 30, troglitazone (30 μM); 31, trichostatin A (1 μM); 32, sodium butyrate (1 mM); 33, phorbol 12-myristate 13-acetate (50 nM). The promoter activity was calculated by dividing the promoter activity of unstimulated cells (column 1). Data are expressed as means ± SE for each group. *P<0.05. *REG*, *regenerating gene*; HNSCC, head and neck squamous cell carcinoma; IL, interleukin; HGF, hepatocyte growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; TNF-α, tumor necrosis factor-α; Dx, dexamethasone; IFN, interferon; SE, standard error; resveratrol, 3,4',5-trihydroxy-*trans*-stilbene.

described (44), which delivered the dose at 0.8 Gy/min. Chemotherapy involved the application of cisplatin (Nihon Kayaku Co., Tokyo, Japan) to the cultures at a concentration of 0, 1.0, 3.0 or 10 μM as described (44).

As for chemo- and radiosensitivity, the cell viability was assessed using WST-8 cleavage. Cells were plated in 96-well plates and incubated for 24 h. Initial density of FaDu cells was $3x10^3$ cells/well, and of HSC-4 cells $1x10^3$ cells/well. After 24 h, 30 μM of resveratrol were added to each well, while DMSO was added as a control, and incubated for 48 h. For radiotherapy, they were then irradiated at 0, 5, or 10 Gy respectively. For chemotherapy, cisplatin (0-10 μM) was added to cultures. Following incubation for additional 72 h, the absorbance of each well at 450 nm (reference wavelength at 620 nm) was read as described above. Each measurement was repeated at least four times on each cell line.

Invasion assay. Invasion assays were performed using 24-well Matrigel-coated Transwells (BD Biosciences, Bedford, MA, USA). A total of $4x10^4$ cells of FaDu and HSC-4 were suspended in 200 μl of serum-free DMEM and placed in the top chambers, and 700 μl of DMEM containing 10% FBS were added to the bottom chambers. After 48 h of incubation at 37˚C, non-invading cells were removed from the top of the Matrigel with a cotton swab, while invading cells on the bottom surface of the filter were fixed in 4% paraformaldehyde and stained with Giemsa (Sigma-Aldrich) for 10 min. The invading cells were then visualized at x200 magnification and counted in five fields for each filter.

Data analysis. Data were expressed as means ± standard error (SE). Statistical significant differences between groups were determined by Student's t-test using StatMate IV (Abacus Concepts, Berkeley, CA, USA). P<0.05 was considered significant.

Results

Activation of REG III gene promoters in FaDu cells. To investigate activators of *REG III* gene expression, we tested various stimulative substances in two clones of FaDu cells to which the *REG III* promoter vector was stably introduced (Fig. 1). Among these various stimulators, polyphenols such as resveratrol, genistein, daidzein, and histone deacetylase inhibitors such as sodium butyrate, significantly increased the *REG III* promoter activity (Fig. 1). We also tested the concentration-response relationship of resveratrol, genistein, daidzein, sodium butyrate for *REG III* promoter

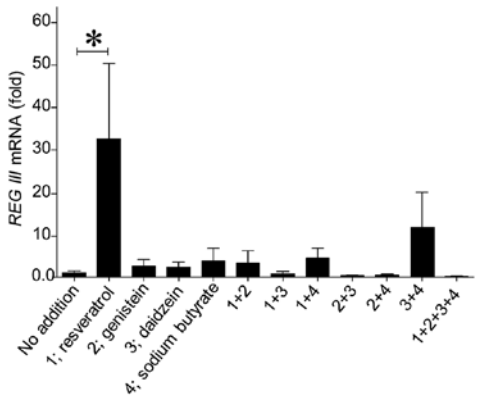

Figure 2. The mRNA levels of *REG III* in FaDu cells treated without (no addition) or with resveratrol (10 μM), or genistein (25 μM), or daidzein (40 μM), or sodium butyrate (2 mM). Data are expressed as the ratio of the number of target mRNA normalized by β-*actin* mRNA as means ± SE for each group (N=4). *P<0.05. *REG*, *regenerating gene*; resveratrol, 3,4',5-trihydroxy-*trans*-stilbene; SE, standard error.

activation, and found that the optimum concentration was 10 μM (resveratrol), 25 μM (genistein), 40 μM (daizein), and 2 mM (sodium butyrate) (data not shown).

Induction of REG III mRNA by candidate substances in FaDu cells. We examined mRNA levels of intrinsic *REG III* in FaDu cells by using real-time RT-PCR, after treating cells with 10 μM resveratrol, 25 μM genistein, 40 μM daidzein, and 2 mM sodium butyrate, respectively. Resveratrol significantly increased the mRNA levels of *REG III* as compared with the others (Fig. 2). The combined addition of resveratrol with other candidates did not enhance, rather inhibited, the increment of *REG III* mRNA expression by resveratrol alone. It might be due to over-stimulation for the signaling pathway, and as a result, it might be higher concentrations for the effect, as reported by Liu *et al* (53). We also tested the concentration (0-100 μM)-response relationship of *REG III* mRNA expression by resveratrol and found that the optimum concentration of resveratrol for *REG III* expression was 30 μM (data not shown).

Effect of resveratrol on growth inhibition in HNSCC cells. We sought to evaluate the effect of resveratrol on cell proliferation of HNSCC cells, FaDu and HSC-4. In the proliferation assay using WST-8 cleavage, HNSCC cells (both FaDu and HSC-4 cells) treated with 30 μM resveratrol showed a significant decrease in growth compared to untreated cells as a control. The resveratrol-induced growth inhibitory effect in HNSCC cells was time-dependent (Fig. 3).

Resveratrol enhances the chemo- and radiosensitivity of HNSCC cells. We measured the chemo- and radiosensitivity by addition of resveratrol to HNSCC cells using WST-8 cleavage. We found that HNSCC cells treated with resveratrol showed a significant increase in chemosensitivity (3.0 and 10 μM of cisplatin) and radiosensitivity (5 and 10 Gy), as compared with cells treated with DMSO as a control (Fig. 4). Therefore, these results indicated that resveratrol enhances the chemo- and radiosensitivity of HNSCC cells.

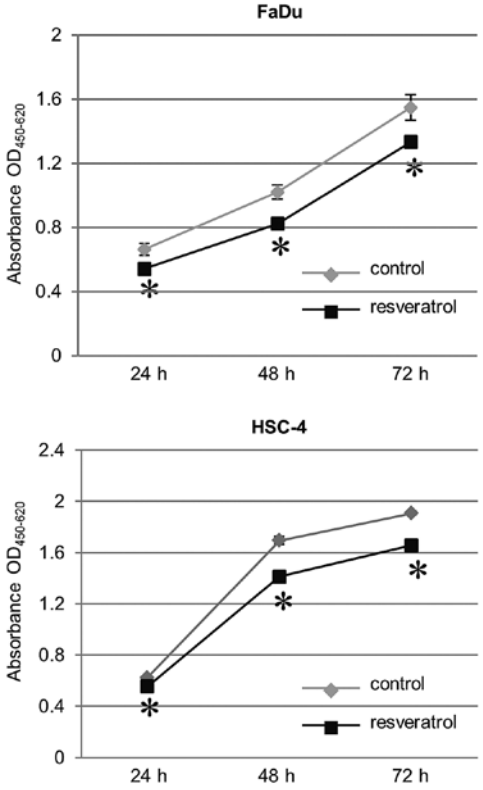

Figure 3. Effect of resveratrol on cell proliferation in HNSCC cells. HNSCC cells treated without (no addition) or with resveratrol (30 μM). Cell proliferation was determined by WST-8 assay at 24 h intervals up to 72 h in FaDu and HSC-4 cell lines. Data are shown as means ± SE. *P<0.05. Resveratrol, 3,4',5-trihydroxy-*trans*-stilbene; HNSCC, head and neck squamous cell carcinoma; SE, standard error.

Resveratrol blocks cancer invasion of HNSCC cells. We measured the invasive ability by addition of resveratrol to HNSCC cells using an invasion assay. We found that cells treated with resveratrol displayed significantly less invasive ability compared to that of the untreated cells as a control (Fig. 5).

Discussion

REG family proteins are believed to be associated with human digestive diseases such as inflammation and cancer (32-42,44,54,55). Recently, among human REG gene family, we have reported that *REG III* expression was associated with an improved survival rate for HNSCC patients, and *REG III* regulated cell proliferation and chemo- and radiosensitivity in HNSCC *in vitro* (44).

REG proteins are upregulated in many human cancers, and play their roles through several signaling pathways. According to previous reports, expression of REG was mainly induced by various inflammatory cytokines and exogenous growth factors (13). However, there is little information on these pathways. Some studies have demonstrated that cytokines such as IFN-γ, IL-6, IL-22, and TNF-α, enhance the expression of *REG I* (46,49,56-58). In addition, it is revealed that IL-8 regulates the expression of REG protein in gastric cancer cells (59) and that *REG I* acts as an anti-apoptotic factor through the STAT3 signaling pathway by increasing the expression and

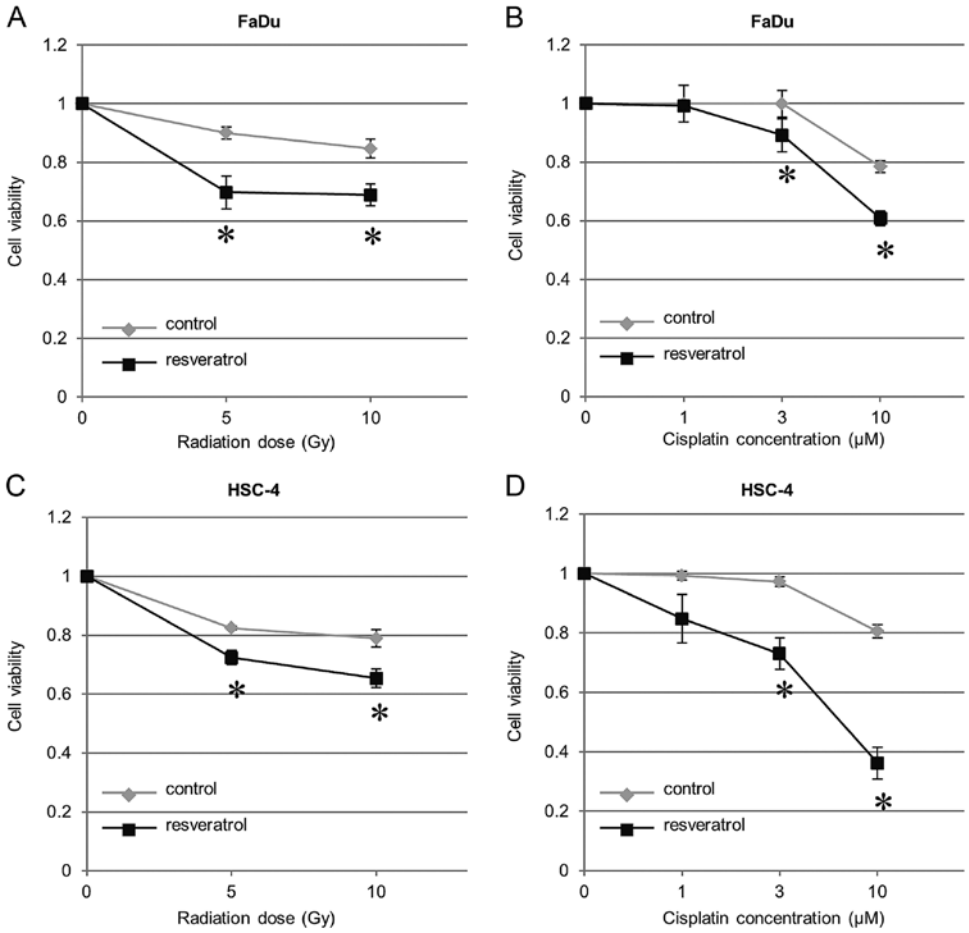

Figure 4. Enhancement of chemo- and radiosensitivity in HNSCC cells by resveratrol. Cells were treated with (A and C) radiation at a dose of 0, 5 or 10 Gy, (B and D) 0, 1.0, 3.0, or 10 μM cisplatin. Thereafter each dish was incubated for additional 72 h. Cell viability was assessed using WST-8 assay. Data are shown as means ± SE. *P<0.05. HNSCC, head and neck squamous cell carcinoma; resveratrol, 3,4',5-trihydroxy-*trans*-stilbene; SE, standard error.

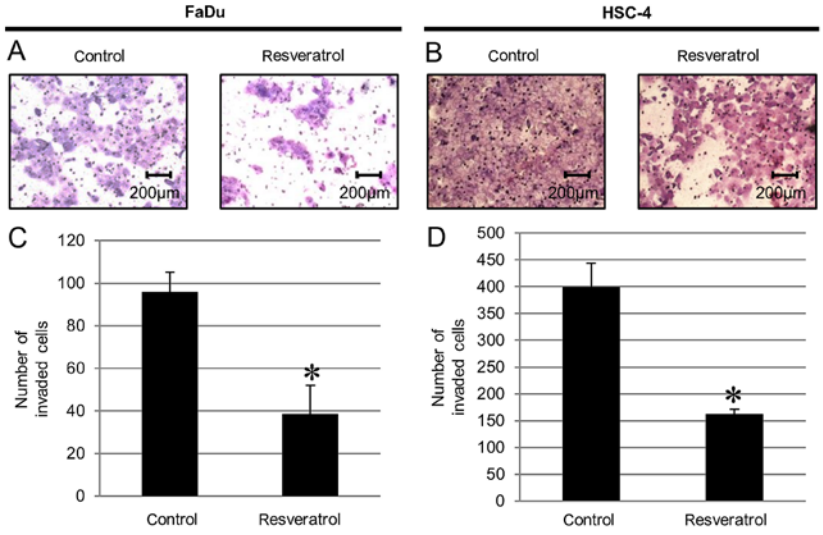

Figure 5. Inhibitory effect of resveratrol on cancer invasion in HNSCC cells. (A and B) Invading cells were visualized at x200 magnification. (C and D) The number of invading cells was counted in five fields for each filter. Results are expressed as means ± SE. *P<0.05. Resveratrol, 3,4',5-trihydroxy-*trans*-stilbene; HNSCC, head and neck squamous cell carcinoma; SE, standard error.

phosphorylation of Akt and Bad in gastric cancer cells (60). Furthermore, some studies demonstrated that PPARγ activator of thiazolidinediones such as ciglitazone and troglitazone inhibited *REG Iα* gene transcription (61). However, the biological function and cell signaling pathway of *REG III* have not been elucidated.

In the present study, we investigated the stimulators for the induction of the expression of *REG III* in HNSCC *in vitro*.

In comparison with other type of *REG* family genes, expression of *REG III* was not induced by inflammatory cytokines, growth factors, and PPARγ activator of thiazolidinediones. We first revealed that resveratrol, which is a naturally occurring polyphenol, significantly increased the *REG III* promoter activity and the mRNA levels of *REG III* in HNSCC cells.

Resveratrol is a natural compound present in fruits including red grapes, vegetables and beverages including wine that are part of the human diet (62-64). In recent years, many studies indicated that resveratrol is associated with antioxidant, anti-inflammatory, and anticarcinogenic effects (53,65,66). Concerning the anticarcinogenic potential of resveratrol, a number of studies have suggested that resveratrol modulates multiple cellular processes, including cell proliferation, apoptosis, inflammation, and angiogenesis (63,67-71). Several reports indicate that resveratrol inhibits proliferation of cancer cells by inhibiting cell cycle progression (72-75). Moreover, recent studies support the role of resveratrol in inhibition of cancer invasion (76,77). In the present study, we observed a reduction in cell growth rates and the enhancement of chemo- and radiosensitivity in HNSCC cells treated with resveratrol when compared with untreated cells as a control. Furthermore, the present study demonstrated that resveratrol blocked cancer invasion in HNSCC cells. These results were compatible to the anticarcinogenic effect in cells transfected with *REG III* (44). It can be presumed that resveratrol could enhance the chemo- and radiosensitivity and inhibit cancer progression through the *REG III* expression pathway in HNSCC cells. However, the downstream signaling pathway of *REG III* is still unresolved. The signaling pathway of how *REG III* reduces cell proliferation, blocks cancer invasion, and enhances the chemo- and radiosensitivity in HNSCC need to be investigated in future studies.

In summary, these data suggested that resveratrol can play an important role in the improvement of survival for HNSCC through the *REG III* expression pathway and can be a potential candidate for novel anticancer drugs for patients with HNSCC.

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