Abstract. Identifying the key molecules that enhance chemo- and radiosensitivity in head and neck squamous cell carcinoma (HNSCC) as well as reliable biomarkers for predicting recurrence and metastasis would be desirable to improve the prognosis of HNSCC. Previously, we have reported that Regenerating gene III (REG III) expression was associated with an improved survival rate for patients with HNSCC. In addition, resveratrol (3,4',5-trihydroxystilbene) significantly increased REG III expression in HNSCC cells, and significantly inhibited cell growth, enhanced chemo- and radiosensitivity, and blocked the cancer invasion of HNSCC cells in vitro. In the present study, the effect of resveratrol on cancer progression in HNSCC was investigated in vivo using a xenograft nude mouse model. The results revealed that resveratrol increased the mRNA level of REG III in vivo, which was in agreement with our previous in vitro findings. Furthermore, REG III increased the antitumor effect of radiation or cisplatin in vivo, and resveratrol sensitized HNSCC to irradiation and cisplatin in vivo. These results indicated that resveratrol could increase the efficacy of cisplatin and irradiation through the REG III expression pathway, resulting in the inhibition of HNSCC progression in vivo.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide (1,2). Cancer in the head and neck region can arise from several functional disorders associated chewing, speech, swallowing and respiration. Therefore, therapy for HNSCC is required to not only cure the disease, but to also preserve the function of the affected area in order to maintain the quality of life of patients with HNSCC. Despite the recent advancements in surgery, chemotherapy and radiotherapy, limited improvements in treating metastatic HNSCC have been achieved (3,4). Two-thirds of all patients present advanced stage III or IV tumors with low locoregional control rates and the long-term survival of patients with HNSCC has remained insufficient (5,6). Therefore, novel agents for potential alternative HNSCC treatments with greater efficacy are urgently required. The present study focused on the human regenerating gene (REG) as a reliable biomarker for predicting HNSCC progression. REG was first identified in regenerating pancreatic islets in studies on diabetology in 1988 (7). REG family proteins are classified into 4 subfamilies: Types I, II, III and IV. The human REG family consists of 5 members: REG Iα, REG Iβ, REG III, hepatocarcinoma-intestine-pancreas/pancreatitis-associated-protein and REG IV (8-13). The REG family of proteins has been revealed to serve roles in normal tissue regeneration (14,15) and also the progression of various types of cancers, including esophageal, gastric, lung, liver, colorectal and prostate cancer (16-25). Recently, we reported that REG III expression was associated with improved survival rates for HNSCC, and that REG III enhanced chemo- and radiosensitivity in vitro (26). In addition, as REG III contributes to the improvement of the prognosis of HNSCC, in our previous study we searched for the substance that induced the expression of REG III and finally revealed that resveratrol (3,4',5-trihydroxy-trans-stilbene) significantly increased REG III expression in HNSCC cells, and also significantly inhibited cell growth, enhanced chemo- and radiosensitivity, and blocked HNSCC cell invasion in vitro (27). The aim of the present study was to investigate the effect of resveratrol on cancer progression in HNSCC in vivo for clinical application.

Materials and methods

Cell culture and reagent. Human hypopharyngeal cell carcinoma FaDu cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and grown...
and maintained in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and supplemented with 100 U/ml penicillin G and 100 µg/ml streptomycin and 250 ng/ml amphotericin B (Antibiotic-Antimycotic; Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated in 5% CO2/95% air with a humidified atmosphere at 37°C.

3,4,5-Trihydroxy-trans-stilbene (resveratrol) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Resveratrol was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and then diluted with normal saline to achieve the correct dose in 300 µl of 2% DMSO.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated using a Rnaprotect® Cell Mini kit (Qiagen GmbH, Hilden, Germany) from FaDu cells. cDNA was reverse-transcribed from 0.5-2 µg samples of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) as previously described (26-33). cDNA was subjected to RT-qPCR with the following primers, which were synthesized and prepared by NGRl (Sendai, Japan): β-actin (NM_001101) sense, 5'-GCGAGAAGATGACCCAGA-3' and antisense, 5'-CGAGGCCGTACAGGGATA-3'; and REG III (AB16037) sense 5'-GAATATTCCTCCCAACTG-3' and antisense, 5'-GAGAAAAGCTGAAATGAAG-3'.

RT-qPCR was performed using the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems; Roche Diagnostics, Indianapolis, IN, USA) and Thermal Cycler Dice Real-Time System (Takara Bio, Inc., Otsu, Japan) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Indianapolis, IN, USA) and supplemented with MIKAMI (Nara, Japan). Each cage housed 3 mice with food and water available ad libitum in a pathogen-free environment with a 12-h light/dark cycle.

**Animals.** BALB/c nude male mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). All protocols were approved by the Animal Care and Use Committee of Nara Medical University (Nara, Japan). Each cage housed 3 mice with food and water available ad libitum in a pathogen-free environment with a 12-h light/dark cycle.

**In vivo model for resveratrol-induced REG III expression in HNSCC cells.** A total of 6 BALB-c nude male mice were used in each experiment. FaDu cells (1x10⁶ cells/100 µl saline) were implanted subcutaneously into the right flanks of BALB/c nude male mice (4-5 weeks old). When the tumors reached ~100 mm³ volume, the mice were randomly assigned to the treatment groups (n=3/group; day 0). For chemosensitivity experiments, the treatment groups consisted of the vehicle control (normal saline) and cisplatin. On day 0, cisplatin (Nihon Kayaku Co., Tokyo, Japan; 4 mg/kg/week) or normal saline was administered intraperitoneally; a total of 4 injections of cisplatin were administered. For the radiotherapy experiments, the treatment groups consisted of vehicle control (normal saline) and 6 Gy irradiation. Mice were exposed to radiation using a MBR-1520R system (Hitachi Ltd., Tokyo, Japan) operated at 150 kV and 20 mA as previously described (27), which delivered the dose at 0.8 Gy/min. For both the chemosensitivity and radiotherapy experiments, the tumor size was assessed every 3 days. The tumor volume was calculated using the following formula: Volume=[Lx(W)²]/2, where L is the length and W is the width. At 24 days post-treatment, the mice were sacrificed with intraperitoneal administration of pentobarbital (100 mg/kg) and the tumor tissues were harvested.

**Statistical analysis.** Data were expressed as the mean ± standard error. Statistically significant differences between groups were determined by Student's t-test using StatMate IV (Abacus Concepts, Piscataway, NJ, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Induction of REG III mRNA by resveratrol in the tumor tissues of a xenograft mouse model.** To elucidate the
induction of REG III in HNSCC cells by resveratrol in vivo, the present study investigated the levels of REG III mRNA in xenografted FaDu cells with or without resveratrol. The mRNA levels of REG III in the resveratrol-treated tumor tissues were significantly increased by resveratrol when compared with the untreated tumor tissues, which were used as the control (Fig. 1). This result was in agreement with the in vitro results we previously reported (27). Therefore, resveratrol induced REG III mRNA expression in vivo as well as in vitro in HNSCC.

REG III enhances the efficacy of radiation or cisplatin therapy in a HNSCC xenograft mouse model. To evaluate the effect of REG III on chemo- and radiosensitivity in HNSCC in vivo, the present study established a HNSCC xenograft model of BALB/c nude mice. An animal xenograft model was generated via injections in nude mice with FaDu cells stably transfected with the REG III expression plasmid (FaDu-REG III) or FaDu cells transfected the neomycin-resistance gene alone (FaDu-mock) as the control into the right flank. When tumors reached ~100 mm³ following 2 weeks, the mice were randomly assigned into 2 groups: Non-treatment, and irradiation or cisplatin therapy groups (day 0), each specific treatment was then applied as aforementioned (Fig. 2A). The efficacy of the radiation or cisplatin therapy was evaluated by monitoring the tumor volume. Regarding the effect of radiation, there were no significant differences in tumor volume between the FaDu-REG III and FaDu-mock in the non-treatment groups. However, in the radiation groups the tumor volume of the FaDu-REG III-treated mice was significantly inhibited when compared with FaDu-mock from day 21 (Fig. 2B-D). Regarding the effect of cisplatin therapy, significant inhibition of tumor progression was observed in the FaDu-REG III group when compared with the FaDu-mock-treated groups from day 15, which corresponded with the results in the radiation groups (Fig. 2B-D). These results indicated that REG III enhanced chemo- and radiosensitivity in HNSCC in vivo as well as in vitro, as previously described (26).
Irradiation or cisplatin therapy with resveratrol synergistically inhibits HNSCC xenograft tumor growth in vivo. To assess the in vivo therapeutic potential of resveratrol, the present study examined tumor progression using a HNSCC xenograft model of BALB/c nude mice. Tumors in the right flanks of the mice were established for 2 weeks prior to the initiation of the treatments, then the mice were randomly assigned into 4 groups for experiments evaluating cisplatin therapy or irradiation, respectively (day 0; Fig. 3A). The potential of the treatment was evaluated by assessing the tumor volume. Regarding the potential of radiation therapy, mice in the resveratrol alone group had smaller tumor volumes than mice in the control group; however, the difference was not statistically significant. Mice in the radiation with resveratrol group had significantly smaller tumor volumes than mice in the control group. In addition, the radiation with resveratrol group exhibited significant antitumor effects when compared with the resveratrol alone or cisplatin alone groups from day 24 (Fig. 3B-D). These results indicated that resveratrol enhanced chemo- and radiosensitivity in HNSCC in vivo, which was in agreement with the effects observed with REG III in vivo.

Discussion

Despite the progression in the current cancer treatments available, such as surgery, radiation and chemotherapy, these have not been effective in improving the survival rate of HNSCC, particularly hypopharyngeal carcinoma (1-6). Chemo- and radioresistance can cause local recurrence and distant metastasis, which are associated with poor prognosis. Therefore, identification of reliable biomarkers that enhance sensitivity for the chemo- and radiotherapy of HNSCC is highly desirable to improve prognosis. As a biomarker of HNSCC, we have focused on REG, whose family of proteins have been associated with diseases such as chronic inflammation and malignant tumors (16-26).

We have previously reported that REG III expression was associated with an improved survival rate for patients with...
Resveratrol, a polyphenolic compound found in grapes and other food products that provides a number of anti-aging health benefits against metabolism, cardiovascular disease and carcinogenesis (34-37). Various previous studies indicated that resveratrol enhanced the sensitivity of chemo-or radiotherapy (38-42). Furthermore, some studies have demonstrated that resveratrol significantly decreased tumor progression (39,40,43). Such in vitro studies on the associations between resveratrol and cancer have been reported (27). This result highlights the potential of resveratrol in inducing the REG III expression in vivo.

In the present study, a HNSCC xenograft nude mouse model was established to evaluate the effect of REG III on cancer progression in HNSCC in vivo. It was demonstrated that REG III increased the antitumor effect of radiation or cisplatin in vivo. In addition, the in vivo therapeutic potential of resveratrol was evaluated, and the results revealed that it significantly sensitized HNSCC to irradiation and cisplatin in vivo, although resveratrol is not likely to be a primary treatment for HNSCC. These results indicate that resveratrol has potential for use as an adjuvant anticancer therapy in HNSCC.

In our previous studies, similar results were obtained in HSC-4 cells as well as FaDu cells (27). In terms of its effect for HNSCC in vivo, further studies whether HSC-4 cells have similar results based on the results of this study are required in the future. Moreover, it is necessary to consider which combination of chemotherapy, radiotherapy and resveratrol is the most effective treatment. The present study performed in vivo experiments using a xenograft mouse model. For clinical applications associated with the administration of resveratrol, experiments on an orthotopic transplant mouse model may be required in order to take into consideration the micro-environment in the future. Concerning the bioavailability of resveratrol, recent studies have indicated that resveratrol induces apoptosis or autophagy in several human cancer cell lines and in an animal model of carcinogenesis (49,50). It has been reported that resveratrol induces cell apoptosis and cell cycle arrest via the caspase/cyclin-CDK signaling pathway (49). The present study investigated the anti-carcinogenic potential of resveratrol by analyzing the REG III expression pathway. Preliminary experiments were performed to investigate the anti-carcinogenic mechanism of resveratrol, by analyzing the expression of apoptosis-related proteins. These preliminary results revealed that both resveratrol and REG III decreased the expression of cyclin D1, B-cell lymphoma-xL and activated caspase-3 compared with the control (data not shown). This indicated that resveratrol may inhibit cancer progression through the REG III pathway via the caspase/cyclin-CDK signaling pathway. However, the underlying mechanism of how resveratrol enhances REG III, and how REG III enhances the chemo- and radiosensitivity of HNSCC remains unknown. Therefore, further studies are required in the future.

In conclusion, the results of the present study indicate that resveratrol increases the efficacy of cisplatin and irradiation through the REG III expression pathway, resulting in the inhibition of tumor growth, in treating HNSCC in vivo. The present study provides support for clinical trials using resveratrol as an adjuvant anticancer therapy and may help improve human HNSCC prognosis. However, additional studies will be required to fully define the therapeutic potential of resveratrol for HNSCC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

IO, SM and ST conceived and designed the research. SM, TM and TU conducted the experiments. SM, HO, TKim and TU were involved in the analysis and interpretation of data. SM wrote the manuscript. IO and SM revised the manuscript for important intellectual content. TKit and IO were involved in editing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All protocols were approved by the Animal Care and Use Committee of Nara Medical University (Nara, Japan). All procedures performed in the studies were in accordance with the 1964 Declaration of Helsinki and its later amendments.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

References


