# Expression of *REG III* and prognosis in head and neck cancer

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**Abstract.** Identification of a reliable biomarker for predicting prognosis in head and neck cancers is highly desirable and has long been sought. There have been several reports that members of the regenerating gene (*REG*) family are highly expressed in chronic inflammation and in tumors of the digestive organs. In addition, it has been described in several reports that *REG* expression is associated with the progression of digestive cancers. In the present study, we evaluated the effect of *REG* expression on the prognosis of hypopharyngeal cancer. We investigated 37 cases with hypopharyngeal squamous cell carcinoma, determined *REG* mRNA expression, which is easily detected in formalin-fixed paraffin-embedded tissues using the real-time reverse transcriptase-polymerase chain reaction method, and evaluated the survival rate using the Kaplan-Meier method. According to these results, *REG III* mRNA expression was significantly associated with prolonged survival. Therefore, we constructed hypopharyngeal cancer cell lines transfected with *REG III* and assessed the cell proliferation and chemosensitivity and/or radiosensitivity *in vitro*. Cells transfected with *REG III* exhibited significantly lower cell proliferation and higher chemosensitivity and/or radiosensitivity compared with the control cells. These data suggest that *REG III* may be a reliable biomarker of prognosis in hypopharyngeal cancer. This is the first report concerning the association of *REG III* expression and the prognosis of head and neck squamous cell carcinoma including hypopharyngeal cancer.

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*Abbreviations: REG*, regenerating gene; HNSCC, head and neck squamous cell carcinoma; CRT, chemoradiotherapy; UC, ulcerative colitis; *HIP/PAP*, hepatocarcinoma-intestine-pancreas/pancreatitis-associated-protein; RT-PCR, reverse transcriptase-polymerase chain reaction; IL, interleukin

*Key words: REG III*, head and neck cancer, chemoradiosensitivity

## Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer in the world and is also known for its rapid clinical progression and poor prognosis. The survival rate for HNSCC patients with advanced stage disease, particularly hypopharyngeal cancer, has improved little over the past 60 years (1,2). Although extended surgery for advanced HNSCC has progressed due to the technique of microsurgery, issues concerning loss of function, aesthetic appearance and risk of various surgical complications remain unresolved. In recent years, definitive chemoradiotherapy (CRT) has become the primary treatment for advanced HNSCC in lieu of surgery, due to the advantage of preserving organ structure and function, and the equivalency in the curative effect compared with surgery. However, the survival rate of HNSCC patients has not significantly improved. Furthermore, CRT can be effective in some patients, while others show little response and experience various adverse effects, which result in the lost of opportunity for a potentially curative surgery. Therefore, we propose that it is important to differentiate whether or not each HNSCC case is chemosensitive and/or radiosensitive prior to treatment. Recently, the human papilloma virus has been identified as one of the biomarkers of chemosensitivity and/or radiosensitivity in oropharyngeal cancers (3,4). In contrast, there is no reliable marker for HNSCC in other sites.

It was previously reported that carcinogenesis is associated with chronic inflammation. Concerning digestive organs, gastritis with *Helicobacter pylori* infection and ulcerative colitis (UC) frequently cause gastric cancer and colorectal cancer, respectively. Recently, there have been many reports that regenerating gene (*REG*) expression is observed in chronic inflammation and in tumors of the digestive organs (5-9). In addition, it has been reported that *REG* expression is associated with progression of digestive cancers such as esophageal, gastric and colorectal cancer (10-16).

By differential screening of the regenerating pancreatic islet-derived cDNA library, *Reg* was found and defined as a regenerating and growth factor (17-20). The *Reg* family belongs to the lectin superfamily and encodes five small secreted proteins (17,21,22). Members of the *Reg* family are grouped into four subtypes: types I, II, III, and IV (23). In humans, the

*REG* family is composed of five subclasses: *REG Iα*, *REG Iβ*, *REG III*, hepatocarcinoma-intestine-pancreas/pancreatitis-associated-protein (*HIP/PAP*) and *REG IV* (5,17,24-29). It has been demonstrated that they are highly expressed in a variety of inflammatory states and in tumor tissue when compared to normal tissue (17,29-31). We hypothesized that *REG* expression is associated with head and neck cancer derived from the oral and pharyngolaryngeal cavities, which belong to the first section of the digestive tract which are exposed to chronic inflammatory factors such as tobacco, alcohol, viral infection and the other various mechanical stress.

In the present study, we extracted RNA from formalin-fixed paraffin-embedded HNSCC tissue, specifically hypopharyngeal cancer, determined mRNA expression of the *REG* family gemes and evaluated the effects of *REG* family expression on the prognosis of hypopharyngeal cancer. The results revealed that *REG III* expression was significantly associated with an increased survival rate. Furthermore, we demonstrated that *REG III* regulated cell proliferation and chemosensitivity and/or radiosensitivity in HNSCC cells *in vitro*.

## Materials and methods

*Study population*. We confirmed 37 cases with hypopharyngeal squamous cell carcinoma. All patients were treated with definitive CRT as a primary treatment between January 2000 and December 2009 at the Department of Otolaryngology-Head and Neck Surgery of Nara Medical University. The present study was approved by the Ethics Committee of Nara Medical University School of Medicine. Written informed consent for participation in the present study was obtained from each patient. The patient characteristics are listed in Table I. The patients included 34 males and 3 females, with a mean age of 68 years (range, 47-83 years). The average period of observation was 34 months (range, 3-98 months).

Table I. Patient and tumor characteristics.

| Characteristics | No. of patients (n=37) |
|---|---|
| Gender | |
| Male | 34 |
| Female | 3 |
| Age (years) | |
| Median | 68 |
| Range | 47-83 |
| Period of observation (months) | |
| Median | 34 |
| Range | 3-98 |
| Tumor stage | |
| I | 4 |
| II | 6 |
| III | 6 |
| IV | 21 |
| T classification | |
| T1 | 8 |
| T2 | 14 |
| T3 | 7 |
| T4 | 8 |
| N classification | |
| N0 | 12 |
| N1 | 7 |
| N2 | 16 |
| N3 | 2 |

T and N classifications are according to the American Joint Committee on Cancer.

*Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)*. Total RNA was isolated from each paraffin-embedded tissue upon biopsy or surgery using the RNeasy FFPE kit (Qiagen, Hilden, Germany). cDNA was then reverse transcribed from ~1 μg samples of total RNA using a High Capacity cDNA reverse transcription kit, with RNase inhibitor (Applied Biosystems, Foster City, CA, USA) as described (32,33). Real-time RT-PCR was then carried out using the primers listed in Table II and SYBR Fast qPCR Master Mix (Kapa Biosystems, Boston, MA, USA). All PCR primers were synthesized by NGRL (Sendai, Japan). PCR was performed with an initial step of 3 min at 95˚C followed by 40 cycles of 3 sec at 95˚C and 20 sec at 60˚C for *β-actin*, *REG III* and *HIP/PAP*, 40 cycles of 3 sec at 95˚C and 20 sec at 64˚C for *REG Iα*, *REG Iβ* and *REG IV*. The level of target mRNA was normalized to the mRNA level of *β-actin* as an internal standard.

*Survival analysis*. We investigated the differences in prognosis between the patient group with positive *REG* family expression and the negative group. Overall survival rate was calculated by the Kaplan-Meier method.

*Cell lines and culture*. FaDu hypopharyngeal squamous cell carcinoma cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and antibiotics (penicillin G/streptomycin/amphotericin B; Gibco) in a humidified incubator at 37˚C.

*Isolation of cells following stable transfection with the REG III expression vector*. cDNA fragment encoding human *REG III* (nucleotides 56-635 of AB161037) was inserted into the pCI-neo mammalian expression vector (Promega, Madison, WI, USA). The expression vector or control vector (without insert DNA) was then introduced into FaDu cells by electroporation using Gene Pulser Xcell™ (Bio-Rad, Hercules, CA, USA) as described (15), after which the cells were cultured in DMEM supplemented with 10% FBS and 500 μg/ml Geneticin® (Invitrogen) for 2 weeks. We determined the *REG III* expression in each cell line transfected with the *REG III* or control vector using real-time RT-PCR method.

*Cell proliferation assay*. Cell proliferation was assessed by Cell Counting Kit-8 (WST-8 cleavage; Dojindo, Mashiki-machi, Japan) as described (15). Cells were seeded

Table II. Primers for real-time RT-PCR.

| Gene | | Primer sequence (position) |
|---|---|---|
| *REG Iα* (NM_002909) | Sense | 5'-AGGAGAGTGGCACTGATGACTT-3' (nucleotides 369-390) |
| | Antisense | 5'-TAGGAGACCAGGGACCCACTG-3' (nucleotides 445-465) |
| *REG Iβ* (NM_006507) | Sense | 5'-GCTGATCTCCTCCCTGATGTTC-3' (nucleotides 108-129) |
| | Antisense | 5'-GGCAGCTGATTCGGGGATTA-3' (nucleotides 170-190) |
| *REG III* (AB161037) | Sense | 5'-GAATATTCTCCCCAAACTG-3' (nucleotides 695-713) |
| | Antisense | 5'-GAGAAAAGCCTGAAATGAAG-3' (nucleotides 765-784) |
| *HIP/PAP* (NM_138937) | Sense | 5'-AGAGAATATTCGCTTAATTCC-3' (nucleotides 645-665) |
| | Antisense | 5'-AATGAAGAGACTGAAATGACA-3' (nucleotides 716-736) |
| *REG IV* (AY007243) | Sense | 5'-ATCCTGGTCTGGCAAGTC-3' (nucleotides 470-487) |
| | Antisense | 5'-CGTTGCTGCTCCAAGTTA-3' (nucleotides 538-555) |
| *β-actin* (NM_001101) | Sense | 5'-GCGAGAAGATGACCCAGA-3' (nucleotides 420-437) |
| | Antisense | 5'-CAGAGGCGTACAGGGATA-3' (nucleotides 492-509) |

RT-PCR, reverse transcriptase-polymerase chain reaction; *REG*, regenerating gene.

in 96-well plates at an initial density of $1x10^3$ cells/well and incubated for 0, 24, 48 or 72 h. Ten microliters of WST-8 solution [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was added to each well, and the plate was incubated for another 2 h. The absorbance of each well at 450 nm (reference wave length at 620 nm) was determined by a Multiscan FC microplate photometer (Thermo Scientific, Waltham, MA, USA). Each measurement was repeated at least eight times on each cell line.

*Radiotherapy and chemotherapy for cultured cells*. Cells were exposed to 0, 4 or 8 Gy irradiation using a MBR-1520R (Hitachi Co., Ibaraki, Japan) operating at 150 kV and 20 mA, which delivered a dose at 0.8 Gy/min. For chemotherapy, cells were treated with cisplatin (Nihon Kayaku Co., Tokyo, Japan) at a concentration of 1.0 or 10 $\mu$M.

In regards to chemosensitivity and/or radiosensitivity, cell viability following chemotherapy, radiotherapy or concurrent CRT in FaDu cells untransfected or transfected with *REG III* was evaluated using WST-8 cleavage. Cells were seeded in 96-well plates at an initial density of $3x10^3$ cells/well and incubated for 24 h. For radiotherapy, they were then irradiated at 0, 4 or 8 Gy. For chemotherapy, cisplatin (0-10 $\mu$M) was added to each well. For concurrent therapy, the cells were irradiated (4 Gy) 2 h after the chemotherapy. Following incubation for an additional 48 h, absorbance at 450 nm (reference wave length at 620 nm) was measured as described above. Each measurement was repeated at least eight times on each cell line.

*Statistical analysis*. Data are presented as means ± standard error (SE). Significant differences between groups were assessed using a log rank test for survival analysis and one-way analysis of variance (ANOVA) with the Dunnett multiple comparison test for *in vitro* study (StatMate III; Abacus Concepts, Berkeley, CA, USA). The differences were considered to be significant at $P<0.01$.

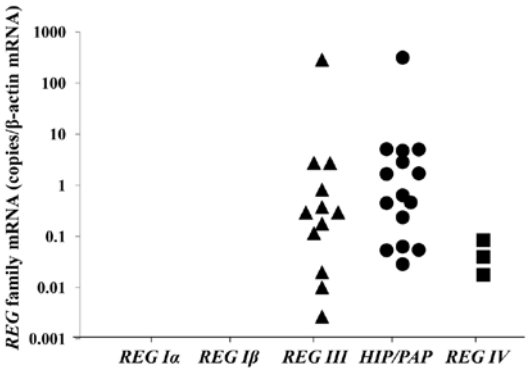


Figure 1. Number of cases expressing mRNA of each regenerating gene (*REG*) family member as determined by real-time reverse transcriptase-polymerase chain reaction using *β-actin* mRNA as an endogenous control.

## Results

*REG family gene expression in hypopharyngeal squamous cell carcinomas*. The mRNA expression of *REG* family genes in each case was measured using real-time RT-PCR. No case with positive expression of *REG Iα* and *REG Iβ* was noted, and only 3 cases were positive for *REG IV* expression, while there were 12 and 15 cases positive for *REG III* and *HIP/PAP* expression, respectively (Fig. 1). No positive case among the normal tissues in the hypopharyngeal area showed expression for any of the *REG* family genes.

*Differences in survival determined by the clinical data*. Each overall survival rate was calculated using the Kaplan-Meier method. The *REG III* expression-positive group showed long-term survival when compared to the negative group with significant difference (Fig. 2A), whereas there were no differences between groups in regards to *HIP/PAP* and *REG IV* expression (Fig. 2B and C). These data suggest that *REG III* expression is associated with a more favorable prognosis of hypopharyngeal squamous cell carcinoma.

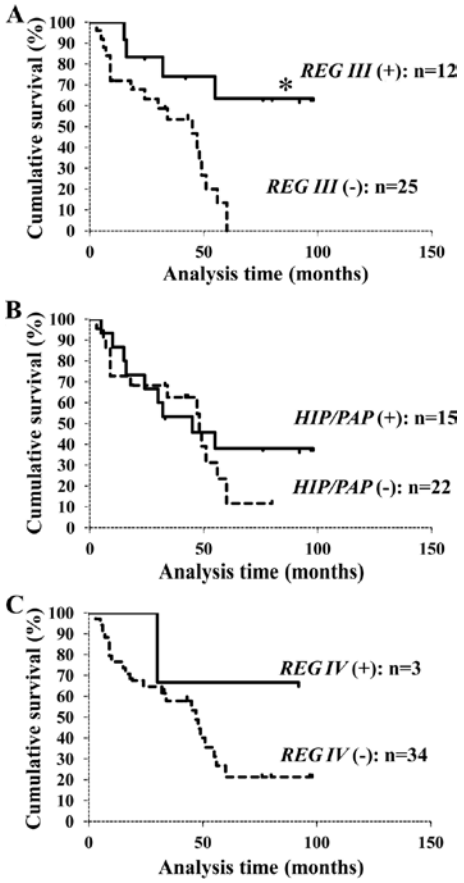


Figure 2. Effects on the survival rate of expression of regenerating gene *(REG) III* (A), hepatocarcinoma-intestine-pancreas/pancreatitis-associated-protein (*HIP/PAP)* (B) and *REG IV* (C). The solid line represents patients with tumors that expressed *REG* family (*REG III*, *HIP/PAP* and *REG IV*) mRNAs whereas the broken line represents patients with tumors that did not express *REG* family mRNAs. *P<0.01 assessed as synergy by log-rank test.

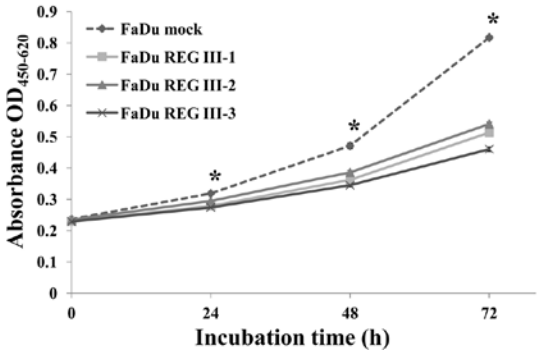


Figure 3. Differences in cell proliferation among the cell lines. FaDu hypopharyngeal squamous cell carcinoma cells were stably transfected with the regenerating gene (*REG*) *III* expression vector (FaDu REG III-1, -2 and -3 cells) or control vector (FaDu mock cells). Cell proliferation was determined by WST-8 assay at 24 h intervals up to 72 h in each FaDu cell line. Data are shown as mean ± SE. *P<0.01.

*REG III suppresses the growth of FaDu cells*. To estimate the effect of *REG III* on hypopharyngeal cancer cell growth, we stably transfected FaDu cells, which originally express very low level of *REG III* mRNA, with an expression plasmid for *REG III*, after which the expression of *REG III* mRNA was assessed (data not shown). FaDu cells transfected with the

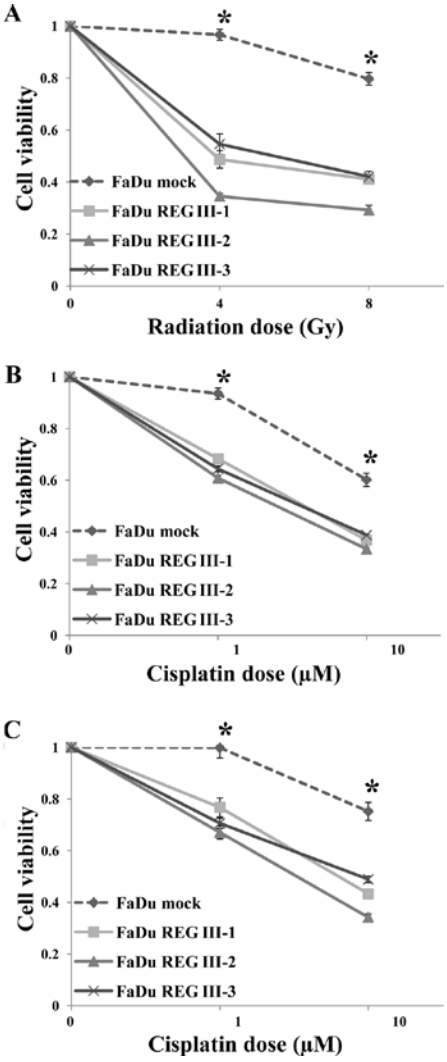


Figure 4. Enhancement of chemoradiosensitivity by regenerating gene (*REG*) *III* expression *in vitro*. All cells were initially incubated for 24 h. For radiotherapy (A), cells were then irradiated at 0, 4 or 8 Gy. For chemotherapy (B) and concurrent therapy (C), cells were treated with various concentrations of cisplatin. For the concurrent therapy, 2 h later the cells were then irradiated at 4 Gy. Thereafter each dish was incubated for an additional 48 h. Cell viability was assessed using WST-8 assay. Data are shown as mean ± SE. *P<0.01.

*REG III* expression plasmid (FaDu REG III-1, -2 and -3 cells) showed higher expression of *REG III* than the cells transfected with the neomycin-resistance gene alone (FaDu mock).

In the cell proliferation assay using WST-8 cleavage, FaDu REG III-1, -2 and -3 cells showed a significant decrease in growth rate when compared with the rate in the FaDu mock cells (Fig. 3).

*REG III enhances the chemosensitivity and/or radiosensitivity of FaDu cells*. FaDu REG III-1, -2 and -3 cells showed a significant increase in radiosensitivity at 4 and 8 Gy and chemosensitivity at 1.0 and 10 $\mu$M cisplatin, as compared with the FaDu mock cells (Fig. 4A and B). Furthermore, chemoradiosensitivity was also significantly higher in the FaDu REG III-1, -2 and -3 cells at 1.0 and 10 $\mu$M cisplatin (Fig. 4C). Thus, these results imply that *REG III* enhances the chemosensitivity and/or radiosensitivity of hypopharyngeal cancer cells.

## Discussion

Members of the *Reg* family are grouped into four subtypes: types I, II, III, and IV; the human *REG* family is composed of five subclasses: *REG Iα*, *REG Iβ*, *REG III*, hepatocarcinoma-intestine-pancreas/pancreatitis-associated-protein (*HIP/PAP*) and *REG IV*. *REG III* and *HIP/PAP* belong to type III. The nucleotide sequence of *REG III* mRNA is very similar to that of *HIP/PAP* mRNA (29). Although there are many reports concerning *REG Iα*, *HIP/PAP* and *REG IV*, little is known regarding *REG III*. *REG III* is strongly expressed in the pancreas, moderately in the testis and weakly in the heart, kidney and placenta, whereas *HIP/PAP* is strongly expressed in the pancreas and small intestine and weakly in hepatoma, stomach, brain and heart (29). Type III Reg proteins as well as type I Reg proteins are suggested to be induced in response to tissue inflammation such as pancreatitis (29,34). However, the details of their biological function have not been fully elucidated.

In the present study, we observed many cases of hypopharyngeal cancer expressing *REG III* or *HIP/PAP*. *HIP/PAP* expression was not associated with a significant difference in survival, while the survival rate of patients with *REG III* expression was significantly prolonged when compared with that of the negative cases. *In vitro*, we also observe a reduction in cell growth rates and the enhancement of chemosensitivity and/or radiosensitivity in the FaDu cells transfected with *REG III* when compared with the control. These outcomes were compatible with the clinical data.

Type III Reg proteins have been suggested to be involved in cellular proliferation of intestinal, hepatic and neuronal cells (35,36). High expression of type III Reg proteins has been observed in carcinomas in digestive organs and inflammatory diseases such as pancreatitis, enterocolitis and UC (29,31,37-39). Furthermore, type III proteins are also present in response to neuron damage and participate in the regeneration of neurons (35,36,40). However, as the details regarding the effects of type III Reg proteins on intracellular signaling remain to be elucidated, it is still unclear how *REG III* functioned to enhance the chemosensitivity and/or radiosensitivity and improve the survival of patients with hypopharyngeal squamous cell carcinoma in the present study.

Several reports have shown that interleukin-6 (IL-6) and dexamethasone activate the transcription of *REG I* (23,34). and that type III is induced by various cytokines, such as IL-6, INF-γ and TNF-α (41,42). To investigate how *REG III* expression is regulated in HNSCC cells, we determined the expression of IL-6, -8 and -11 using real-time RT-PCR method using clinical samples in the present study. The expression of *REG III* and IL-11 had no correlation, while the expression of IL-6 and IL-8 had a positive correlation with the expression of *REG III* (data not shown). Although the details are still unknown, these results indicate the possibility that IL-6 and IL-8 can become key factors to elucidate the relationship between the expression of *REG III* and the prognosis of hypopharyngeal cancer patients.

It has been demonstrated that *Reg* is highly expressed in regenerating islets and tissues of pancreatitis, whereas this expression declines when the function of the pancreas is improved (17,37,43,44). Moreover, *in vivo*, transfection with *Reg* into a normal rat caused neither proliferation of β-cells nor hyperplasia of islets (19). These results suggest that there is an unknown suppressive function *in vivo* in contrast with the proliferative activity by *Reg* expression. In the present study, it was also expected that *REG III* may act as a suppression factor with various functions in hypopharyngeal cancer.

These data suggest that *REG III*, which can be easily detected in formalin-fixed paraffin-embedded tissues with RT-PCR analysis, may be a reliable biomarker of the chemosensitivity and/or radiosensitivity and prognosis of hypopharyngeal cancer. However, the biological function and cell signaling pathway of *REG III* require further elucidation. The critical mechanisms warrant further investigation. This is the first report concerning the association between *REG III* expression and the chemosensitivity and/or radiosensitivity and prognosis of HNSCC including hypopharyngeal cancer.

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