Site-specific methylation patterns of the *GAL* and *GALR1/2* genes in head and neck cancer: potential utility as biomarkers for prognosis

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ABSTRACT

Background: The aim of this study was to evaluate the prognostic value of the promoter methylation status of galanin (*GAL*) and galanin receptor 1/2 (*GALR1/2*) by assessing their association with disease-free survival and known prognostic factors in head and neck cancer.

Methods: We generated methylation profiles of *GAL* and *GALR1/2* in tumor samples obtained from 202 patients with head and neck squamous cell carcinoma (HNSCC); these included 43 hypopharynx, 42 larynx, 59 oral cavity, and 58 oropharynx tumor samples. CpG island hypermethylation status of the 3 genes was analyzed using quantitative methylation-specific PCR (Q-MSP). In order to determine the prognostic value of the methylation status of these genes, the associations between methylation index and various clinical characteristics, especially tumor site, were assessed for tumors from patients with HNSCC.

Results: The methylation index was positively correlated with female gender (P = 0.008) and disease recurrence (P = 0.01) in oral cancer and human papillomavirus (HPV)-positive (*P* = 0.004) status and disease recurrence (P = 0.005) in oropharyngeal cancer. Among patients with oral and oropharyngeal cancer, promoter hypermethylation of *GAL*, *GALR1*, or *GALR2* was statistically correlated with a decrease in disease-free survival (log-rank test, P = 0.036 and P = 0.042, respectively). Furthermore, methylation of *GAL*, *GALR1*, or *GALR2* exhibited the highest association with poor survival (log-rank test, *P* = 0.018) in patients with HPV-negative oropharyngeal cancers.

Conclusions: As such, *GAL* and *GALR1/2* methylation status may serve as an important site-specific biomarker for prediction of clinical outcome in patients with HNSCC.

INTRODUCTION

Head and neck squamous cell carcinomas (HNSCCs) constitute an anatomically heterogeneous group of solid tumors arising from the nasopharynx, oral cavity, oropharynx, hypopharynx, and larynx [1]. In addition, HNSCC is a highly heterogeneous disease that develops via one of two primary routes: chemical carcinogenesis through exposure to tobacco and alcohol or virally induced tumorigenesis [2,3]. Over the last few decades, there has been a decline in carcinomas of the hypopharynx and larynx [4]. In contrast to this trend, the incidence of oropharynx squamous cell carcinomas (OPSCCs) has increased over the last couple of decades [5]. Human papillomavirus (HPV)-associated OPSCCs represent distinct disease entities in terms of their epidemiology, biology, and clinical behavior relative to their tobacco-associated counterparts [6]. Therefore, molecular classification of HNSCCs is required to provide prognostic as well as mechanistic information to improve patient care.

Aberrant promoter methylation, an important hallmark of cancer cells, is considered a major mechanism underlying the inactivation of tumor-related genes. Several studies have reported

that promoter methylation of tumor suppressor genes represents a common mechanism of transcriptional silencing in HNSCC [3]. Aberrant methylation in several tumor suppressor genes has been demonstrated to be involved in the development and progression of HNSCC and has been used as a biomarker to definitively predict disease outcome[7]. Numerous epigenetic events in carcinogenic pathways have been studied recently, resulting in the development of methods for detecting CpG island promoter methylation patterns to stratify high-risk groups among patients with HNSCC. Choudhury reported that promoter methylation of *DAPK*, *p16*, *RASSF1*, and *MINT31* is significantly associated with HPV (+) tumors of HNSCC [8]. Promoter hypermethylation of *DAPK* and *p16* is significantly associated with smoking status and may be used to predict the risk of incidence of HNSCC [9]. The degree of global hypomethylation in HNSCC is associated with smoking history, alcohol consumption, and tumor stage [10].

Our recent efforts to determine the methylation profiles of *GAL* and *GALR1/2* were insufficient because of the small sample size studied and lack of discrimination between the sites of origin of primary tumors [11,12]. However, it is likely that the galanin system plays a dominant role in tumorigenesis in HNSCC. G protein-coupled receptors (GPCRs) modulate multiple intracellular signaling transduction pathways and elicit cytostatic and/ or cytotoxic effects, which include cell cycle arrest and apoptosis [13]. Furthermore, epigenetic repression of GPCR expression is related to prognosis and the response to radiotherapy/chemotherapy [14]. Although previous studies have revealed a correlation between high-methylation tumors and decreased survival, this finding requires external validation along with site-specific analysis.

The aim of this study was to determine the methylation status of *GAL*, *GALR1*, and *GALR2* in HNSCC to evaluate their clinical significance as prognostic biomarkers for recurrence risk and survival. We attempted to determine whether HNSCC primary tumors originating from different anatomic sites (hypopharynx, larynx, oral cavity, and oropharynx) exhibited similar DNA methylation changes, or whether DNA methylation events were specific to the anatomic site.

RESULTS

Analysis of methylation status of *GAL* and *GALR1/2* genes

Q-MSP was used to assess the aberrant promoter methylation status of *GAL*, *GALR1*, and *GALR2* in tumors from the hypopharynx (n = 43), larynx (n = 42), oral cavity (n = 59), or oropharynx (n = 58). *GAL* was methylated in 5 (11.6%), *GALR1* in 19 (44.2%), and *GALR2* in 13 (28.6%) of the 43 hypopharyngeal cancers examined. In laryngeal cancers, the frequency of hypermethylation was 23.8% for *GAL*, 59.5% for *GALR1*, and 40.5% for *GALR2*. The frequency of promoter methylation was detected to be 19.0% for *GAL*, 60.3% for *GALR1*, and 32.8% for *GALR2* in oropharyngeal cancers. Among 59 cases with oral cancers, the frequency of hypermethylation was 20.3% for *GAL*, 40.7% for *GALR1*, and 45.8% for *GALR2* (Fig. 1A). Analysis of the 43 hypopharyngeal samples revealed that at least one of these three genes was methylated in 18 (65.1%) primary tumors (Fig. 1B). The frequency of methylation of at least one gene was increased in laryngeal cancers (66.7%) (Fig. 1C), oral cancers (59.3%) (Fig. 1D), and oropharyngeal cancers (70.7%) (Fig. 1E). Matched pairs of head and neck tumors and adjacent normal mucosal tissues were obtained from surgical specimens collected from 67 patients for initial methylation screening. We have added these data as Supplementary Table 2.

Correlation between *GAL* and *GALR1/2* methylation and clinicopathological assessment

Characteristics and clinicopathologic features of patients, including age at diagnosis, sex, alcohol consumption, smoking habit, tumor staging, HPV status, and tumor recurrence, are summarized in Table 1. Methylation index (MI) was defined as the ratio between the number of methylated genes and the total number of tested genes in each sample. The mean differences in MI according to the age of onset, sex, alcohol consumption, smoking habit, tumor size, lymph node

status, clinical stage, HPV status, and recurrence are illustrated in Fig. 2. In particular, in hypopharyngeal cancers, MI was significantly higher in male (0.98 +/- 0.77) than in female (0.29 +/- 0.49; P = 0.030) patients (Fig. 2A). There was no significant association between clinicopathologic characteristics in 35 laryngeal cancer patients (Fig. 2B). HPV-positive oropharyngeal cancers show an affinity for the oropharynx (27/58; 46.6%). Among oral cancers, MI was significantly higher in female (1.75 +/- 1.06) than in male (0.89 +/- 0.94; P = 0.001) patients, as well as recurrence-positive cases (1.5 +/- 1.06) relative to recurrence-negative cases (0.81 +/- 0.91; P = 0.010) (Fig. 2C). Notably, we found that MI was significantly higher in HPV-positive than in HPV-negative cases (1.48 +/- 0.64 vs. 0.81 +/- 1.05; P = 0.005), as well as in recurrence-positive cases (1.60 +/- 0.88) relative to recurrence-negative cases (0.87 +/- 0.87; P = 0.004) (Fig. 2D) of oropharyngeal cancers.

Kaplan-Meier estimates

Kaplan-Meier plots indicated that the methylation status of *GAL* and *GALR1/2* was related to disease-free survival (DFS) (Fig. 3A-D). Among 43 patients with hypopharyngeal cancers, the rate of DFS in those with any methylated genes was 19.3% (mean survival time; 27.0 months) compared with 38.1% (mean survival time; 31.2 months) in the group with no methylated genes (log-rank test, P = 0.744) (Fig. 3A). Among the patients with laryngeal cancers, the rate of DFS of patients exhibiting methylation of one or more of the three genes was 24.6% (mean survival time; 27.8 months), compared with 68.6% (mean survival time; 41.6 months) in the group with no methylated genes (log-rank test, P = 0.265) (Fig. 3B). Among 59 cases of oral cancers, the rate of DFS was lower in the any-methylated genes group than in the no-methylated genes group (36.7% vs. 76.1%, respectively; log-rank test, P = 0.035) (Fig. 3C). Among patients with oropharyngeal cancers, the DFS rates for those with no methylated genes and any methylated genes were 85.7% (mean survival time; 22.7 months) and 25.6% (mean survival time; 19.7 months), respectively (log-rank test, P = 0.042) (Fig. 3D). We did not observe any correlation between mortality and HPV status (log-rank

test, P = 0.826) in the cohort of patients with oropharyngeal cancer (Fig. 4A). Overall survival tended to be better in HPV-positive patients than in HPV-negative individuals; however, this was not statistically significant (87.1% versus 53.5%, log-rank test, P = 0.156) (data not shown). However, hypermethylation of any of the 3 investigated genes was significantly associated with shortened survival in HPV- negative patients (log-rank test, P = 0.018) (Fig. 4B).

Prognostic value of *GAL* and *GALR1/2* promoter hypermethylation

The odds of recurrence associated with methylation of *GAL* and *GALR1/2* were estimated by multivariate logistic-regression analysis (Fig. 5A-D). When *GAL* was methylated in laryngeal cancers, the adjusted odds ratio for recurrence was 16.5 (95% confidence interval [CI], 1.84 to 148.29; P = 0.012) (Fig. 5B). In patients with oral cancers, concomitant methylation of the gene pair *GAL* and *GALR1* and the gene pair *GALR1* and *GALR2* was associated with an odds ratio for recurrence of 5.45 (95% CI, 1.48 to 20.1; P = 0.011) and 4.86 (95% CI, 1.26 to 18.7; *P* = 0.021), respectively (Fig. 5C). Notably, patients with oropharyngeal cancers that exhibited methylation of *GALR2* and the gene pair *GAL* and *GALR2* had a significantly higher odds ratio for recurrence of 4.84 (95% CI, 1.34 to 17.53; P = 0.016) and 6.58 (95% CI, 1.67 to 25.89; P = 0.007), respectively (Fig. 5D).

Additional analysis of the 42 patients with laryngeal cancer revealed that those with patients with unmethylated *GAL* showed significantly better DFS in comparison to those with methylated *GAL* (log-rank test, P = 0.021) (Supplemental Fig. 1A). In oral cancers, *GALR1* promoter hypermethylation was statistically correlated with a decrease in DFS (log-rank test, P = 0.008) (Supplemental Fig. 1B). A trend towards poorer DFS was observed in patients with oropharyngeal cancers that exhibited methylation of the *GALR2* promoter (log-rank test, P = 0.052) (Supplemental Fig. 1C).

External validation of results from the TCGA database

The validation of TCGA data for *GAL* and *GALR1/2* methylation in HNSCC and its correlation in cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) cohorts are shown in Fig. S2. Interestingly, *GAL* methylation demonstrated an average β value of 0.328 in the oropharyngeal SCC TCGA cohort and 0.275 in the TCGA CESC subset (Supplemental Fig. 2A). The β value for methylation of *GALR1* was identified in 0.451 in the oropharyngeal SCC TCGA cohort and 0.546 in CESC samples (Supplemental Fig. 2B). The β value of *GALR2* was significantly higher in CESC than in the other HNSCCs ($P < 0.001$, Student's t test) (Supplemental Fig. 2C).

DISCUSSION

The identification of epigenetic modifications of *GAL*, *GALR1*, and *GALR2* genes is important for the elucidation of mechanisms underlying tumorigenesis and for the assessment of recurrence risk in patients. Here, we reported a real-time PCR analysis of DNA methylation profiles in genomic DNA from 202 HNSCC tissues derived from cancers originating in 4 anatomic sites. Overall, we found that aberrant promoter methylation patterns of *GAL* and *GALR1/2* in primary tumors are indicators of an increased risk of recurrence in patients with oral and oropharyngeal cancer. The features of DNA methylation are loci-related, site-specific, as well as correlated with the HPV status of the patient.

Exposure to several carcinogens, such as HPV, tobacco, and alcohol, has been associated with epigenetic gene inactivation in human cancers, e.g. those of the head and neck, esophagus, and lung [15,16]. Recently, oncogenic viruses such as HPV and EBV have been shown to evoke cancerous changes to the DNA methylome of the cell by increasing activity of DNA methyltransferases (DNMTs), enzymes that methylate the DNA of the host genome as part of the tumorigenic pathway [17,18]. Promoter hypermethylation studies have largely identified only a limited number of candidate genes in HNSCC [1]. Therefore, the development of an integrated

analysis method, applicable to various tumor types, is necessary for the discovery of correlation between the tumor primary site and tumor-specific characteristics. Interestingly, we found a strong association between *GAL* and *GALR1/2* methylation levels and gender in hypopharyngeal and oral cancers: however, *GAL* and *GALR1/2* methylation levels were not associated with gender in laryngeal and oropharyngeal cancers. Female gender is positively correlated with methylation for some genes, including *MTAP*, in gastric cancer [19], *CDH1* in lung cancer tissue [20] and *p14* in colorectal cancer [21]. The activity of sex hormones may be mediated via gene-specific epigenetic modifications [22].

GPCRs are the largest signal-conveying receptor family that mediate multiple physiological processes; however, their role in tumor biology is poorly understood [19]. Various studies suggest that possess potent antitumor effects and neuropeptides function as tumor suppressor genes in human cancers. Hypermethylation of the tachykinin-1 (*TAC1*) gene is related with poor prognosis in patients with head and neck, colon, and esophageal cancer [20-22]. The promoter methylation profiles of the *TAC1* and tachykinin receptor 1-encoding gene appear to represent significant markers of outcome in patients with head and neck cancer [20]. In addition, somatostatin promoter hypermethylation is a common event in human colon cancer [22]. Simultaneous analyses of the methylation status of multiple tumor suppressor genes are important for predictions of tumorigenesis, biological behavior, and the development of future targeted therapy.

GAL, a 30-amino acid peptide in humans, has been shown to act as a highly specific and efficient pharmaceutical agent in vivo; GAL targets the galanin system via its cognate receptors GALR1, GALR2, and GALR3 [23]. The identification of *GALR1* methylation in DNA obtained from postmenopausal women indicates the presence of endometrial malignancy [24]. Hypermethylation of *GALR2* has been reported in several cancers such as colorectal cancer [25] and breast cancer [26], as well as in hepatocellular tumorigenesis [27]. The silencing function of either *GAL* or *GALR1* induces the apoptosis of both drug-sensitive and drug-resistant cells and synergistically enhances the effect of

chemotherapy (5-FU and oxaliplatin) in colorectal cancer [28]. Furthermore, *GALR2*-overexpressing colorectal cancer cells are more susceptible to bevacizumab than control cells, and exogenous *GALR2* expression results in the apoptosis of neuroblastoma cells [29,30]. Therefore, our understanding of the functions of *GAL* and *GALR1/2* with respect to HNSCC is improving.

A previous study in our laboratory reported the function of the GALR1/2 signaling pathways in HNSCC [14]. Importantly, the activation of the GALR1 signaling pathway suppresses tumor cell growth via phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), which is related to the downregulation of cyclin D1 and the upregulation of cyclin-dependent kinase inhibitors [31]. The reduction of HNSCC cell growth in response to GALR2 expression in the presence of galanin is due to the induction of apoptosis. GALR2-mediated apoptosis is caspase-independent and involves the downregulation of ERK1/2 and the induction of the pro-apoptotic Bcl-2 protein Bim [32]. While the function of GALR3 is not fully known, GALR3-expressing cells show activation of the PI3K pathway [33]. The *GALR3* promoter region is C + G-rich; however, the degree of condensation of CpG sequences in this region is low [34]. Therefore, the role of GALR3 differs from that of GALR1

and GALR2 [31]. The differing signal transduction pathways related to each galanin receptor might account for their different biological activities in various types of cancer [35]; therefore, the effect of galanin signaling is likely to be dependent on the expression level of each receptor, and to occur in a cell type-specific manner. Significantly, the different methylation patterns of these three genes in primary tumors may be utilized as the basis for identification of patients with an increased risk of recurrence.

A study of this type involving human specimens and utilizing high-throughput profiling platforms may be susceptible to measurement bias from a variety of sources. First, numerous genes have been reported as individual biomarkers for prognosis in HNSCC. The present study provides evidence that the methylation status of *GAL*, *GALR1*, and *GALR2* represents an independent prognostic factor for DFS in patients with oral cancers and HPV-negative oropharyngeal cancers. Further investigations showed the aberrant methylation of *GAL* may be of potential use as a marker for patients with laryngeal cancer that are at a high risk of relapse. Biomarker discovery for HPV-negative HNSCC is crucial for the improvement of patient outcomes. Simultaneous analyses of the methylation status of multiple tumor suppressor genes are important for predictions of tumorigenesis and biological behavior as well as for the development of targeted therapy. Our findings suggest that such methylation markers could be used in clinical practice to distinguish patients that may benefit from adjuvant therapy after initial surgical treatment; however, additional prospective studies are required to validate these genes in other groups of patients with HNSCC.

In conclusion, *GAL* and *GALR1/2* genes were identified as aberrantly methylated in HNSCC patients. Importantly, the methylation patterns of these three genes in primary tumors may be used to identify patients with oral and oropharyngeal cancers that are at a higher risk of recurrence. These findings should benefit oral and oropharyngeal cancer screening and surveillance programs. The differences in promoter methylation patterns observed between HPV-positive and HPV-negative

tumors, and their effects on downstream signaling pathways involved in carcinogenesis, provide several testable hypotheses for further research.

MATERIALS AND METHODS

Tumor samples

Two hundred and two primary HNSCC samples were obtained from patients during surgery at the Department of Otolaryngology, Hamamatsu University School of Medicine. All patients provided written informed consent and the study protocol was approved by the Institutional Review Board of the Hamamatsu University School of Medicine. Clinical information, including age, sex, tumor site, smoking habit, alcohol consumption, tumor size, lymph node status, and stage grouping were obtained from the patients' clinical records. The male: female ratio of the patients was 171: 31. The mean age was 64.9 years (range = 36-90). Primary tumors were located in the hypopharynx (n = 43), larynx (n = 42), oral cavity (n = 59), or oropharynx (n = 58).

Bisulfite treatment and quantitative methylation-specific PCR analysis

Genomic DNA was extracted using the MethylEasy Xceed Rapid DNA Bisulfite Modification Kit (TaKaRa, Tokyo, Japan) and subjected to bisulfite conversion, as previously described [11]. The methylation status of the CpG islands in the promoter region of *GAL* and *GALR1/2* was determined in 202 primary HNSCC samples and 67 noncancerous mucosal samples. Promoter methylation levels of *GAL* and *GALR1/2* were determined using quantitative methylation-specific PCR (Q-MSP) with the TaKaRa Thermal Cycler Dice TM Real Time System TP800 (TaKaRa, Tokyo, Japan). The primer sequences are listed in Supplemental Table 1. A standard curve was established using serial dilutions of EpiScope^{TM} Methylated HeLa gDNA (TaKaRa, Tokyo,

Japan). The normalized methylation value (NMV) was determined as follows: NMV = (Target gene-S/Target gene-FM)/(ACTB-S/ACTB-FM), where Target gene-S and Target gene-FM represent target gene methylation levels in the sample and universal methylated DNA, respectively, and ACTB-S and ACTB-FM correspond to β-actin in the sample and universal methylated DNA, respectively. Analysis was performed using the Thermal Cycler Dice Real Time System TP800 Software Ver. 1.03A (TaKaRa, Tokyo, Japan), according to the manufacturer's directions for use [12].

Analysis of high-risk HPV status

The HPV status was evaluated using the HPV Typing Set (Takara Bio., Tokyo, Japan), a PCR primer set specifically designed to identify HPV genotypes -16, -18, -31, -33, -35, -52, and -58 in genomic DNA. The PCR HPV Typing Set method was performed according to the manufacturer's protocol. The PCR products were separated using 9% polyacrylamide gel electrophoresis and stained with ethidium bromide.

Collection of publicly available data from The Cancer Genome Atlas (TCGA)

Aberrant DNA methylation data for a total of 522 HNSCC cases, comprising 10 hypopharynx cases, 116 larynx cases, 325 oral cavity cases, 71 oropharynx cases, and 303 CESC cases (TCGA public data available in March 2016), were collected from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga). DNA methylation data obtained using the Infinium HumanMethylation450 platform (Illumina, Inc. CA) were shown as the β value.

Statistical analysis

Statistical analysis for the association of variables was performed using Student's t-test. The disease-free time was measured from the date of the initial treatment to the date of diagnosis of locoregional recurrence or distant metastasis. The Kaplan–Meier test was used to calculate the survival probability, and the log-rank test was used to compare the difference between survival rates. Multivariate logistic regression analysis involving stage grouping, age, sex, alcohol intake, smoking status, and DNA methylation status was used to identify the predictive value of the prognostic factors [36]. Differences with $P < 0.05$ were considered significant. All statistical analyses were performed using StatMate IV (ATMS Co., Ltd., Tokyo, Japan).

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FIGURE CAPTIONS

Figure 1. Summary of the promoter methylation status of *GAL*, *GALR1*, and *GALR2* in 202 HNSCC samples (A) Comparison of rate of methylation status of the promoters of 3 genes (*GAL*, *GALR1*, and *GALR2***)** in patients with hypopharyngeal cancer; blue box, laryngeal cancer; red box, oral cancer; purple box, oropharyngeal cancer; green box. **(B)** Distribution of *GAL*, *GALR1*, and *GALR2* promoter methylation in hypopharyngeal cancer: the promoters of all 3 genes were hypermethylated in 2% (1 of 43) of the tumors, those of 2 genes were hypermethylated in 16% (7 of

43) of the tumors, and those of one gene were hypermethylated in 47% (20 of 43) of the tumors. None of the genes exhibited hypermethylation in 35% (35 of 43) of the tumors. **(C)** Hypermethylation status of genes in tumors from patients with laryngeal cancer (N = 42), **(D)** hypermethylation status of genes in tumors from patients with oral cancer (N = 58), and **(E)** hypermethylation status of genes in tumors from patients with oropharyngeal cancer (N = 59).

Figure 2. Association between methylation indices (MI) and selected clinical parameters The mean MI for the various groups was compared using Student's *t*-tests. Association between MI and selected epidemiologic and clinical characteristics **(A)** hypopharyngeal cancer: statistically significant differences were found for the associations between MI and sex; **(B)** laryngeal cancer: no differences were noted with regard to any of the clinical characteristics; **(C)** oral cancer: statistically significant differences were found for the associations between MI and sex and MI and recurrence status (positive vs. negative); **(D)** oropharyngeal cancer: statistically significant differences were found for the associations between MI and HPV status (positive versus negative) and between MI and recurrence status (positive versus negative). Means and standard deviations are also indicated, and statistical comparisons between groups are depicted. A probability of < 0.05 (*$P < 0.05$) was considered to represent a statistically significant difference.

Figure 3. Kaplan-Meier survival curves for patients with HNSCC according to *GAL*, *GALR1*, and *GALR2* methylation status (A) Methylation status in patients with hypopharyngeal cancer (n = 43; $P = 0.744$) **(B)** Methylation status in patients with laryngeal cancer (n = 42; P = 0.265) **(C)** Methylation status in patients with oral cancer (n = 59; P = 0.036) **(D)** Methylation status in patients with oropharyngeal cancer (n = 58; P = 0.042)

Figure 4. Kaplan-Meier survival curves for patients with oropharyngeal cancer according to HPV status and methylation status (A) HPV status of patients with oropharyngeal cancer (n = 58; P = 0.826); HPV(+), HPV positive; HPV(-), HPV negative. **(B)** Combined analyses of HPV status and *GAL*, *GALR1*, and *GALR2* methylation status (P = 0.018); Me, methylation; Um, unmethylation

Figure 5. Odds ratios for recurrence based on the multivariate logistic-regression model adjusted for age (70 years & older vs. < 70 years), sex, smoking status, alcohol exposure, and tumor stage (I, II, III, or IV) Multivariate logistic-regression analysis revealed the estimated odds of recurrence associated with *GAL*, *GALR1*, and *GALR2* methylation; * P < 0.05. **(A)** Multivariate logistic regression analysis for hypopharyngeal cancer, **(B)** multivariate logistic regression analysis for laryngeal cancer, **(C)** multivariate logistic regression analysis for oral cancer, and **(D)** multivariate logistic regression analysis for oropharyngeal cancer.

Supplemental Figure 1. Kaplan-Meier survival curves (A) *GAL* methylation status in cases of laryngeal cancer, **(B)** *GALR1* methylation status in cases of oral cancer, and **(C)** *GALR2* methylation status in cases of oropharyngeal cancer

Supplemental Figure 2. DNA methylation data from the TCGA database

The DNA methylation data for **(A)** *GAL*, **(B)** *GALR1*, **and (C)** *GALR2* in two cancer types [HNSCC and Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma] were collected from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) in March 2016.

Table 1. Clinical characteristics of recruited head and neck cancer patients.
Patient and tumor characteristics

Age	
70 and older	16 (37%)
Under 70	27 (63%)
Gender	
Male	36 (84%)
Female	7 (16%)
Alcohol exposure	
Ever	36 (84%)
Never	7 (16%)
Smoking status	
Smoker	33 (77%)
Non smoker	10 (23%)
Tumor size	
T1	1 (2%)
T2	19 (44%)
T3	9 (21%)
T4	14 (33%)
Lympho-node status	
N0	12 (28%)
N+	31 (72%)
Stage	
I	0 (0%)
II	8 (19%)
III	8 (19%)
IV	27 (62%)
HPV status	
Positive	-
Negative	-
Recurrence events	
Positive	21 (%)
Negative	22 (%)

Hypopharygx (n = 43)

Larynx
(n = 42)
13 (31%)
29 (69%)
41 (98%)
1 (2%)
33 (79%)
9 (21%)
31 (74%)
11 (26%)
6 (14%)
6 (14%)
9 (22%)
21 (50%)
21 (50%)
21 (50%)
6 (14%)
3 (7%)
8 (19%)
25 (60%)
-
-
17 (40%)
25 (60%)
Oral cavity
(n = 59)
14 (24%)
45 (76%)
47 (80%)
12 (20%)
39 (66%)
20 (34%)
45 (76%)
14 (24%)
11 (19%)
30 (50%)
4 (7%)
14 (24%)
31 (53%)
28 (47%)
8 (14%)
16 (27%)
10 (17%)
25 (42%)
2 (3%)
57 (97%)
22 (37%)
37 (63%)
Oropharyx
(n = 58)
18 (31%)
40 (69%)
47 (81%)
11 (19%)
41 (71%)

17 (29%)
40 (69%)
18 (31%)
11 (19%)
22 (38%)
6 (10%)
19 (33%)
23 (40%)
35 (60%)
7 (12%)
10 (17%)
6 (10%)
35 (60%)
27 (47%)
31 (53%)
20 (34%)
38 (66%)

Table 1. Clinical characteristics of recruited head and neck cancer patients.

Patient and tumor characteristics

Age
70 and older
Under 70
Gender
Male
Female
Alcohol exposure
Ever
Never
Smoking status
Smoker
Non smoker
Tumor size
T1
T2
T3
T4
Lympho-node status
N0
N+
Stage
I
II
III
IV
HPV status
Positive

Negative	
Recurrence events	
Positive	
Negative	
Hypopharygx	
(n = 43)	
16 (37%)	
27 (63%)	
36 (84%)	
7 (16%)	
36 (84%)	
7 (16%)	
33 (77%)	
10 (23%)	
1 (2%)	
19 (44%)	
9 (21%)	
14 (33%)	
12 (28%)	
31 (72%)	
0 (0%)	
8 (19%)	
8 (19%)	
27 (62%)	
-	
-	
21 (%)	
22 (%)	
Larynx	
(n = 42)	
13 (31%)	
29 (69%)	
41 (98%)	
1 (2%)	
33 (79%)	
9 (21%)	
31 (74%)	
11 (26%)	
6 (14%)	
6 (14%)	
9 (22%)	
21 (50%)	
21 (50%)	
21 (50%)	
6 (14%)	
3 (7%)	
8 (19%)	
25 (60%)	
-	
-	
17 (40%)	
25 (60%)	
Oral cavity	
(n = 59)	
14 (24%)	

45 (76%)
47 (80%)
12 (20%)
39 (66%)
20 (34%)
45 (76%)
14 (24%)
11 (19%)
30 (50%)
4 (7%)
14 (24%)
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28 (47%)
8 (14%)
16 (27%)
10 (17%)
25 (42%)
2 (3%)
57 (97%)
22 (37%)
37 (63%)
Oropharyx
(n = 58)
18 (31%)
40 (69%)
47 (81%)
11 (19%)
41 (71%)
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27 (47%)
31 (53%)
20 (34%)
38 (66%)

Site-specific methylation patterns of the *GAL* and *GALR1/2* genes in head and neck cancer: potential utility as biomarkers for prognosis

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Supplemental Table 1. Quantitative methylation-specific PCR primer list

Gene	Forward/Reverse	Base pairs	Sequence (5’-3’)
Galanin	Forward	82	TGACGCGATTTCGGGCGGTT
	Reverse		TATCCGCCGCCCGATATAAC
GALR1	Forward	99	GGTTCGCGGTATTCGGTAGT
	Reverse		GGTTCGCGGTATTCGGTAGT
GALR2	Forward	119	CGATTGCGGGGGTTGGAGTTCGGA
	Reverse		CCAACAACGACCGACGACGCTA
ACTB	Forward	133	TGGTGATGGAGGAGGTTTAGAAGT
	Reverse		AACCAATAAAACCTACTCCTCCCTTAA

Supplementary Table 2. Galanin, GALR1, and GALR2 Gene Methylation Status in 67 Matched Pairs of Tumor and Adjacent Normal Mucosal Tissues.

	Methylation status								
	Galanin			*GALR1*			*GALR2*		
	methylation	unmethylation	*P*-value†	methylation	unmethylation	*P*-value†	methylation	unmethylation	*P*-value†
Tumor (67)	16	51		43	24		36	31	
Normal (67)	0	67	< 0.001	10	57	< 0.001	6	61	< 0.001

†Fisher's exact probability test. * P < 0.05