Original Article

Tumor Suppressor Activity and Inactivation of Galanin Receptor Type 2 by Aberrant Promoter Methylation in Head and Neck Cancer

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BACKGROUND: There is accumulating evidence that *galanin* receptors (GALRs) may be tumor suppressors in head and neck squamous cell carcinoma (HNSCC). Promoter methylation status and gene expression were assessed in a large panel of head and neck primary tumors, based on the hypothesis that cytosine-guanine dinucleotide (CpG) hypermethylation might silence the *galanin* receptor 2 (*GALR2*) gene. **METHODS:** *GALR2* expression was examined in a panel of cell lines by using quantitative reverse transcription polymerase chain reaction (RT-PCR). The methylation status of the *GALR2* promoter was studied using quantitative methylation-specific PCR (Q-MSP). UM-SCC-1 was stably transfected to express *GALR2*. **RESULTS:** *GALR2* expression was suppressed in UM-SCC cell lines, whereas nonmalignant cell lines exhibited stable expression. *GALR2* methylation found in 31 of 100 (31.0%) tumor specimens was significantly correlated with the methylation status of both *GALR1* and *Galanin*. The observed *GALR2* promoter hypermethylation was statistically correlated with a decrease in disease-free survival (log-rank test, $P = .045$). A multivariate logistic-regression analysis revealed a high odds ratio for recurring methylation of *GALR2* and the gene pair *GALR2* and *Galanin*, 8.95 (95% confidence interval, 2.29-35.03; $P = .024$) and 9.05 (95% confidence interval, 1.76-46.50; $P = .008$), respectively. In addition, exogenous expression of *GALR2* suppressed cell proliferation in UM-SCC-1 cells with hypermethylated *Galanin* and *GALR2*-proficient cell lines. **CONCLUSIONS:** Frequent promoter hypermethylation in association with prognosis, and growth suppression after re-expression, supports the hypothesis that *GALR2* may act to suppress tumor activity. *GALR2* is a potentially significant therapeutic target and prognostic factor for this cancer type. ***Cancer* 2014;120:205–13**. © *2013 American Cancer Society.*

KEYWORDS: *GALR2*, *GALR1*, *Galanin*, cytosine-guanine dinucleotide islands, hypermethylation, head and neck cancer.

INTRODUCTION

Galanin receptors are members of the G protein–coupled receptor (GPCR) superfamily encoded by *GALR1*, *GALR2*, and *GALR3*. Binding of the peptide galanin activates these receptors and stimulates numerous signal transduction and integration pathways.[1] Recent findings support a growth regulatory function for *GALR1* because antibody blocking of the receptor enhances proliferation of head and neck squamous cell carcinoma (HNSCC) cells, and *Galanin* and *GALR1* induce a marked and prolonged ERK1/2 activation, up-regulation of $p27^{Kip1}$ and $p57^{Kip2}$, down-regulation of cyclin D1, and consequent inhibition of cell proliferation.[2] Furthermore, expression-negative squamous cell cancers and cell lines exhibit hypermethylation of cytosine-guanine dinucleotide (CpG) islands in the *Galanin* and *GALR1* promoter region.[3,4] Moreover, *Galanin* and *GALR2* induce up-regulation of $p27^{Kip1}$ and $p57^{Kip2}$ and cyclin D1 down-regulation. These effects are similar to *GALR1* signaling, but *GALR2* also induces caspase-3–dependent apoptosis, which is not observed with *GALR1*.[5] Furthermore, other recent studies have shown that reintroduction of *GALR2* into solid tumor cells, including neuroblastoma and pheochromocytoma cells, increases susceptibility to galanin-induced apoptosis and growth inhibition.[6,7] Collectively, these studies on the functions of *Galanin*, *GALR1*, and *GALR2* have led to improved understanding of cancer.

Aberrant DNA methylation patterns can be useful to assess clinical outcomes. We recently defined the methylation profiles of *Galanin* and *GALR1* in HNSCC tumors, at the time of diagnosis and during subsequent follow-up, and

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evaluated the significance of methylation for prognosis and as a biomarker for risk of recurrence.[3,4] Hypermethylation of *GALR2* has been described in several cancers, including colorectal cancer, breast cancer,[8] and hepatocellular carcinoma.[9] However, these studies did not include a clinical association study. The purpose of this study was to first define a *GALR2*, *GALR1*, and *Galanin* methylation profile in HNSCC tumors analyzed at the time of diagnosis and to evaluate its significance in prognosis as well as its value as a biomarker for recurrence.

Results of this study indicate that loss of *GALR2* expression is associated with hypermethylation of the promoter region. Moreover, assessment of tumor specimens confirmed that hypermethylation is common in patient tumors and is associated with *GALR2* methylation status and disease-free survival (DFS). Finally, the restoration of *GALR2* expression in HNSCC cells resulted in the inhibition of colony formation in response to *Galanin* stimulation, thereby supporting the hypothesis that this molecule plays a tumor-suppressive role in HNSCC. Therefore, *GALR2* could be inactivated via promoter hypermethylation in human head and neck cancer cells and tumors, and hypermethylation of *GALR2* could be a critical event in the genesis of HNSCC.

MATERIALS AND METHODS

Tumor Samples and Cell Lines

Tumor specimens were obtained during surgery from 100 primary HNSCC samples. All patients were treated at the Department of Otolaryngology, Hamamatsu University School of Medicine. Clinical information was obtained from clinical records. The mean age was 63.9 years (range, 39-90 years), and the male:female ratio was 78:22. Matched pairs of head and neck tumor and adjacent normal mucosal tissues were obtained from the surgical specimens of 36 patients for initial methylation screening. DNA and complementary DNA (cDNA) from 12 UM-SCC cell lines were kindly provided by Dr. Thomas E. Carey of the University of Michigan and were validated by genotyping in his laboratory.

Bisulfite Modification, Quantitative Methylation-Specific Polymerase Chain Reaction Analysis, and Immunohistochemistry

Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wis). Bisulfite modification of genomic DNA was carried out as described.[3] Promoter methylation levels of *GALR2* were determined using quantitative methylation-specific polymerase chain reaction (Q-MSP) with the TaKaRa Thermal Cycler Dice Real Time System TP800 (TaKaRa, Tokyo, Japan). The Q-MSP primers for methylated DNA were Q-MSP-*GALR2*-F (5′-CGATTGCGGGG GTTGGAGTTCGGA-3′) and Q-MSP-*GALR2*-R (5′-CCAACAACGACCGACGACGCTA-3′), and Q-MSP-*ACTB*-F (5′-TGGTGATGGAGGAGGTTTAG AAGT-3′) and Q-MSP-*ACTB*-R (5′-AACCAATAAAACCTACT CCTCCCTTAA-3′). A standard curve was generated using serial dilutions of EpiScope Methylated HeLa genomic DNA (TaKaRa). The normalized methylation value (NMV) was defined as follows: NMV = *(GALR2-S/GALR2-FM)/(ACTB-S/ACTB-FM)*, where *GALR2-S* and *GALR2-FM* represent *GALR2* methylation levels in the sample and universal methylated DNAs, respectively, whereas *ACTB-S* and *ACTB-FM* correspond to β-actin in the sample and universal methylated DNAs, respectively. To analyze the methylation status of *GALR1*,[3] *Galanin*,[4] *p16*,[10] *RASSF1A*,[11] *MGMT*,[12] *DAPK*,[12] and *DCC*[13] primers, conditions as described previously were used. In addition, vascular endothelial growth factor (VEGF) and cyclin D1 immunohistochemistry analysis was carried out as described.[3,14,15]

Direct Bisulfite Sequencing Analysis for GALR2

Bisulfite-treated DNA was amplified by bisulfite sequencing PCR (BSP) with a pair of primers that were specific for modified upper-strand DNAs but did not contain any CpG sites in their primers. The primer set was 5′-GTTTGGTTAGTTTAGAATTT-3′ (forward) and 5′-AACCTTACCTCATCTAA AC-3′ (reverse). The PCR products were 217 base pairs (bp) in length. Direct bisulfite sequencing analysis was carried out as described.[3]

RNA Extraction and Quantitative-RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and treated with Ribonuclease-Free deoxyribonuclease (DNase; Qiagen). The cDNA was generated from DNase-treated total RNA using Random primers (Invitrogen) with Superscript II reverse transcriptase (Invitrogen). Primers were designed as follows: *GALR2* forward, 5′-CGACCTGTGTTTCATCCTGTG-3′ and *GALR2* reverse, 5′-GGTAGCGGATGGCCAGATA-3′; *GAPDH* forward, 5′-GCACCGTCAAGGCTGAGAAC-3′ and *GAPDH* reverse, 5′-TGGTGAAGACGCCAGTGGA-3′. For comparisons between samples, the messenger RNA (mRNA) expression of the target genes was normalized to *GAPDH* mRNA expression.

Reactivation of GALR2 *Expression*

At 1 hour after plating, cultures were incubated for 48 hours with 5-azacytidine (15 μg/mL, 30 μg/mL; Sigma)

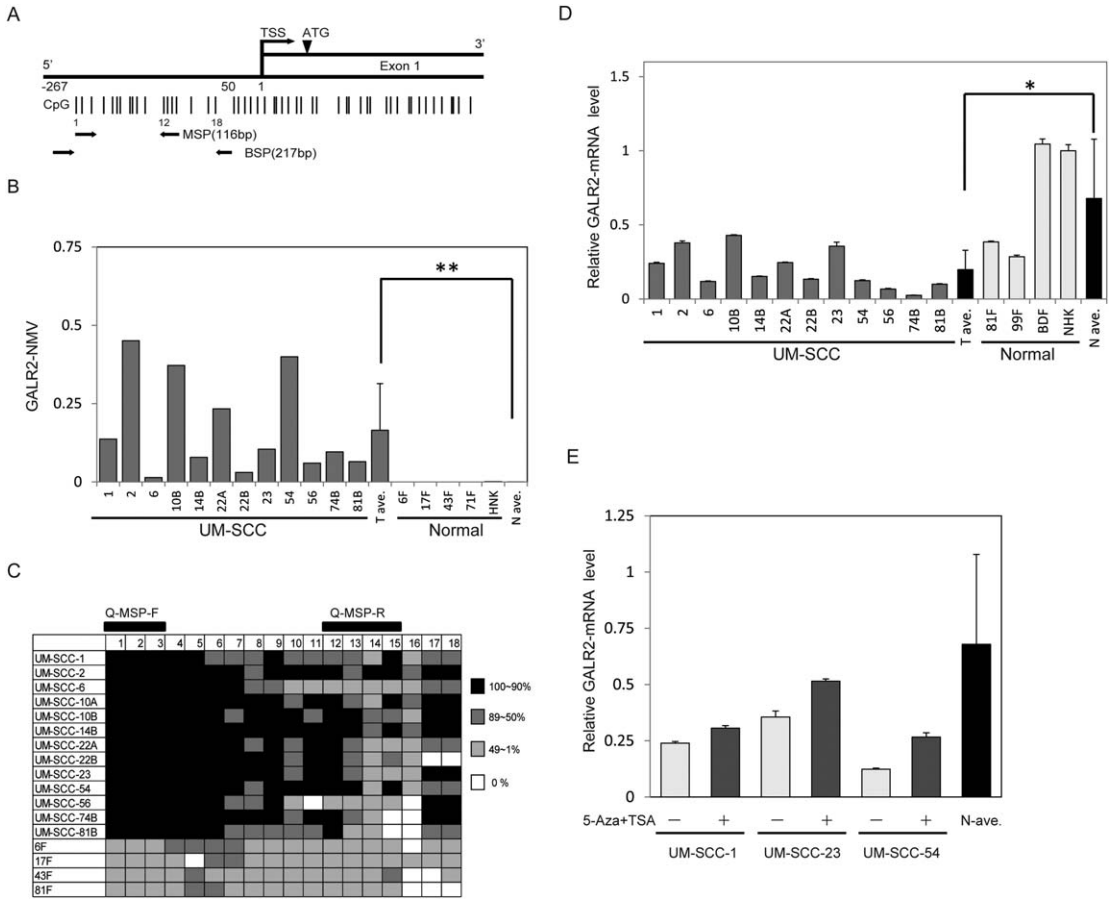

Figure 1. Diagrammatic representation of the *GALR2* gene methylation analysis using the quantitative methylation-specific PCR (Q-MSP) assay, expression by quantitative reverse transcription polymerase chain reaction (Q-RT-PCR), and bisulfite sequencing analysis in UM-SCC cell lines. (A) *GALR2* CpG sites within expanded views of the promoter region relative to the transcription start site (TSS). (B) Representative examples of Q-MSP of *GALR2* in UM-SCC, indicating normal cell lines that are highly methylated (normalized methylation value [NMV] > 0.038) (UM-SCC) or unmethylated (NMV < 0.038). $^{**}P < .01$. (C) Summary of direct bisulfite sequencing analysis of *GALR2* in UM-SCC cell lines. The numbers on the top row indicate the CpG dinucleotide (labeled 1-18 in panel A) in the amplicon. (D) Relative messenger RNA (mRNA) expressions as assessed by Q-RT-PCR of *GALR2* in 12 UM-SCC cell lines and normal cell lines. $^{*}P < .05$. (E) The effect of treatment with 5-azacytidine (5-AZA) and TSA on *GALR2* expression.

followed by 24 hours with 300 nM Tricostatin A (TSA; Sigma).[3]

Plasmid Constructs and Growth Suppression Analysis

UM-SCC-1 was cultured in Dulbecco's modified Eagle medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum at 37°C in 5% CO_2. The *GALR2* sequence was obtained from a human cDNA library (Invitrogen), hemagglutinin-tagged on the COOH terminal end, and subcloned into the pcDNA3 vector (Invitrogen) containing an internal ribosomal entry site (Ires) and green fluorescent protein (GFP) sequence. The pCMVIresGFP vector was used as a transfection control. The UM-SCC-1-GALR2 and UM-SCC-1-mock cells were established by transfecting with pCMVGALR2-HAIresGFP or pCMVIresGFP, respectively, using LipofectAMINE (Invitrogen), followed by selection for GFP-positive cells. At 24 hours after plating, stably transfected cells were fed with serum-free medium containing 0.1% bovine serum albumin for 24 hours to induce quiescence, and then 1 μmol/L galanin (Anaspec) was added. Cell proliferation was measured by counting cells with a Coulter counter model Z1 (Beckman Coulter). Immunoblotting and immunocytochemistry analyses were carried out as described.[5]

Data Analysis and Statistics

Receiver operating characteristic (ROC) curve analysis was performed using the NMV for the 36 HNSCC and

36 adjacent normal mucosal tissues by StatMate IV (ATMS Co, Ltd, Tokyo, Japan). By using this approach, the area under the ROC curve identified optimal sensitivity and specificity levels at which the normal tissues could be distinguished from HNSCC tissues, and corresponding NMV thresholds were calculated for *GALR2*. The cutoff value determined from this ROC curve was applied to determine the frequency of *GALR2* methylation in this study.

For frequency analysis in contingency tables, statistical analyses of associations between variables were performed by Fisher's exact test. In the colony-formation assay, comparisons and tests for statistical significance were made by the student *t* test. The disease-free interval was measured from the date of the treatment to the date when locoregional recurrence or distant metastasis was diagnosed. DFS probabilities were estimated by the Kaplan-Meier method, and the log-rank test was applied to assess the significance of differences among actuarial survival curves. Multivariate logistic-regression analysis was used to determine the predictive value of the prognostic factors.[16,17] Global methylation value is defined as the ratio between the number of methylated genes to the total number of examined genes in each sample. A significant difference was identified when the probability was less than 0.05. Statistical analysis was performed using StatMate IV (ATMS Co, Ltd).

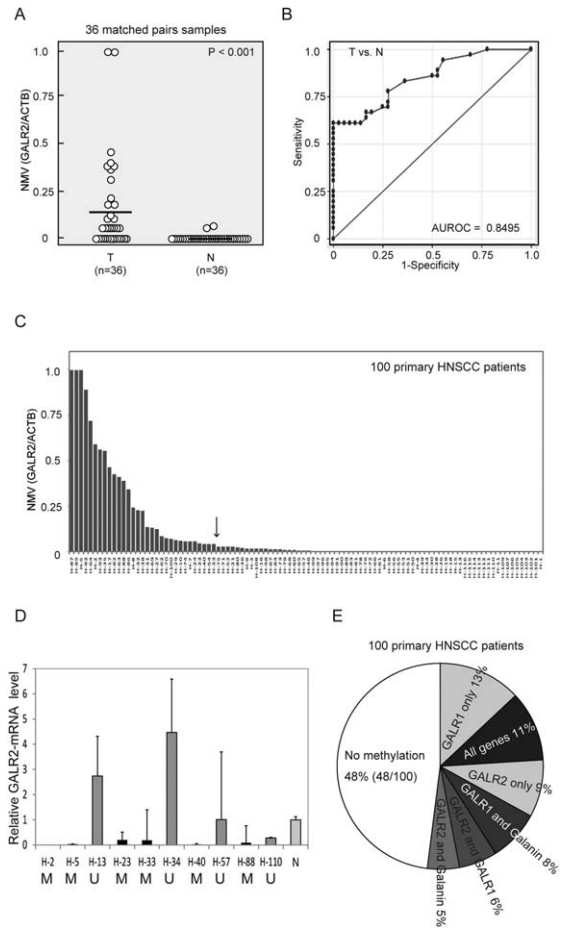

Figure 2. *GALR2* methylation analysis using the quantitative methylation-specific PCR (Q-MSP) assay in head and neck squamous cell carcinoma (HNSCC) tumor samples. (A) Pattern of hypermethylation observed in matched pairs of head and neck tumors and adjacent normal mucosal tissues. (B) Receiver operator characteristic (ROC) curve analysis of normalized methylation value (NMV). AUROC indicates area under the ROC curve. (C) DNA samples from 100 untreated primary tumors tested with the same primers. The location of the NMV cutoff for *GALR2* (0.038) used for the data is indicated by a straight arrow. (D) Relative *GALR2* messenger RNA (mRNA) expression as assessed by Q-RT-PCR in 10 tumor specimens that were also evaluated for *GALR2* promoter methylation. Specimens H-2, H-5, H-23, H-33, H-40, and H-88 exhibited promoter methylation (M), but H-13, H-34, H-57, and H-110 did not (U). (E) Percentage of 100 HNSCC patients with epigenetic alteration in *GALR2*, *GALR1*, and *Galanin* genes, and relative frequency of gene hypermethylation events.

RESULTS

UM-SCC Cell Lines

Forward primers for Q-MSP contained 1 to 3 CpG dinucleotides. Reverse primers for Q-MSP included 12 to 15 CpG dinucleotides (Fig. 1A). In all cancer cell lines with reduced expression, the NMV of *GALR2* was significantly higher than in normal cell lines (Fig. 1B). Direct bisulfate DNA sequencing was carried out to assess the percentage of CpG alleles methylated in the *GALR2* 5′ region. The percentage of methylated alleles for each of the 18 different CpG sites is summarized in Figure 1C for 12 UM-SCC cell lines. The concordance between mRNA expression levels and aberrant methylation of *GALR2* was nearly identical (Fig. 1C). We analyzed 12 UM-SCC cell lines for mRNA expression of *GALR2* by using quantitative RT-PCR and found lower expression in cancer cell lines than in normal cell lines (Fig. 1D).

To establish that methylation was responsible for silencing of the *GALR2* gene expression, we evaluated 3 cell lines (UM-SCC-1, UM-SCC-23, and UM-SCC-54). These cell lines, that exhibited absence of *GALR2* expression and hypermethylation, were cultured with 5-azacytidine plus TSA. The results show that *GALR2* expression was up-regulated after treatment (Fig. 1E).

Initial Screening: Matched Pairs of Head and Neck Tumors and Adjacent Normal Mucosal Tissues

Initially, *GALR2* promoter methylation status was analyzed in 36 cancerous and paired noncancerous mucosae

TABLE 1. *GALR2* Gene Methylation Status in HNSCC Primary Samples

Patient and Tumor Characteristics (n = 100)	Methylation		
	Present (n = 31)	Absent (n = 69)	P Value[a]
Age, y			
70 and older (29)	8	21	
Under 70 (71)	23	48	.812
Sex			
Male (78)	20	58	
Female (22)	11	11	1
Smoking status			
Smoker (69)	19	50	
Nonsmoker (31)	12	19	1
Alcohol exposure			
Ever (58)	17	43	
Never (42)	14	26	1
Tumor size			
T1-2 (49)	12	37	
T3-4 (51)	19	32	.198
Lymph node status			
N0 (44)	13	31	
N+ (56)	18	38	.830
Stage			
I, II, III (45)	13	32	
IV (55)	18	37	.828
VEGF expression			
High expression (52)	16	36	
Low expression (48)	15	33	1
Cyclin D1 expression			
High expression (33)	12	21	
Low expression (67)	19	48	1
p16 methylation			
Yes (54)	20	34	
No (46)	11	35	.195
RASSF1A methylation			
Yes (22)	5	17	
No (78)	26	52	.438
MGMT methylation			
Yes (34)	12	22	
No (66)	19	47	1
DAPK methylation			
Yes (39)	19	20	
No (61)	12	49	.004[b]
E-cadherin methylation			
Yes (40)	15	25	
No (60)	16	44	1
H-cadherin methylation			
Yes (37)	20	17	
No (63)	11	52	.0003[b]
DCC methylation			
Yes (58)	22	36	
No (42)	9	33	.085
COL1A2 methylation			
Yes (52)	22	30	
No (48)	9	39	.017[b]
GALR1 methylation			
Yes (38)	17	21	
No (62)	14	48	.027[b]
Galanin methylation			
Yes (24)	16	8	
No (76)	15	61	6.43×10^{-5}[b]

[a] Fisher's exact probability test.
[b] $P < .05$.

by using Q-MSP. Promoter methylation levels were represented by NMVs, which is the ratio of methylated DNA at the target sequence in each specimen to a fully methylated control DNA. The *GALR2* methylation level was significantly higher in primary HNSCCs than in noncancerous mucosal tissues (median NMV = 0.154 versus 0.08, $P < .001$; Wilcoxon matched pairs test and paired student t test; Fig. 2A).

GALR2 promoter hypermethylation exhibited highly discriminative ROC curve profiles, which clearly distinguished HNSCC from normal mucosal tissues (AUROC = 0.8495). The ROC curve with corresponding areas under the curve for *GALR2* of HNSCC versus normal mucosal tissues is presented in Figure 2B. The cutoff NMV for *GALR2* (0.038) was chosen from the ROC curve to maximize sensitivity (61.1%) and specificity (100%) (Fig. 2B).

Clinicopathological Characteristics of 100 Primary HNSCC Samples

Among 100 DNA samples from previously untreated primary tumors tested with the same primers, a specimen was classified as methylated when its NMV exceeded 0.038. The *GALR2* promoter was methylated in 31 of 100 (31.0%) cases and unmethylated in 69 (69.0%) (Fig. 2C). *GALR2* expression analyzed by Q-RT-PCR identified 6 tumors, namely, H-2, H-5, H-23, H-33, H-40, and H-88 with strong methylation signals, which exhibited little mRNA expression. In contrast, 4 tumors (H-13, H-34, H-57, and H-110) without *GALR2* promoter methylation exhibited relatively robust mRNA expression (Fig. 2D). Methylation of *GALR2* significantly correlated with *DAPK* ($P = .004$), *H-cadherin* ($P = .0003$), *COL1A2* ($P = .017$), *GALR1* ($P = .027$), and *Galanin* methylation status ($P = 6.43 \times ^{-5}$) ; Table 1). Specifically, *GALR1* exhibited promoter hypermethylation in 38% (38 of 100) and *Galanin* in 24% (24 of 100) of the tumors.[3,4] A representative methylation analysis for *GALR2*, *GALR1*, and *Galanin* in tumors is shown in Figure 2E. Eleven percent (11 of 100) of the tumors included all 3 hypermethylated genes: 19% had 2 hypermethylated genes, 22% had only a single hypermethylated gene, and 48% had no methylated gene (Fig. 2E).

Prognostic Value of the GALR2 *and Promoter Methylation of Other Genes*

We found that the presence of *GALR2* promoter methylation was associated with a statistically significant decrease in DFS (log-rank test, $P = .0445$) (Fig. 3A). Methylation of both *Galanin* and *GALR2* was associated with a DFS

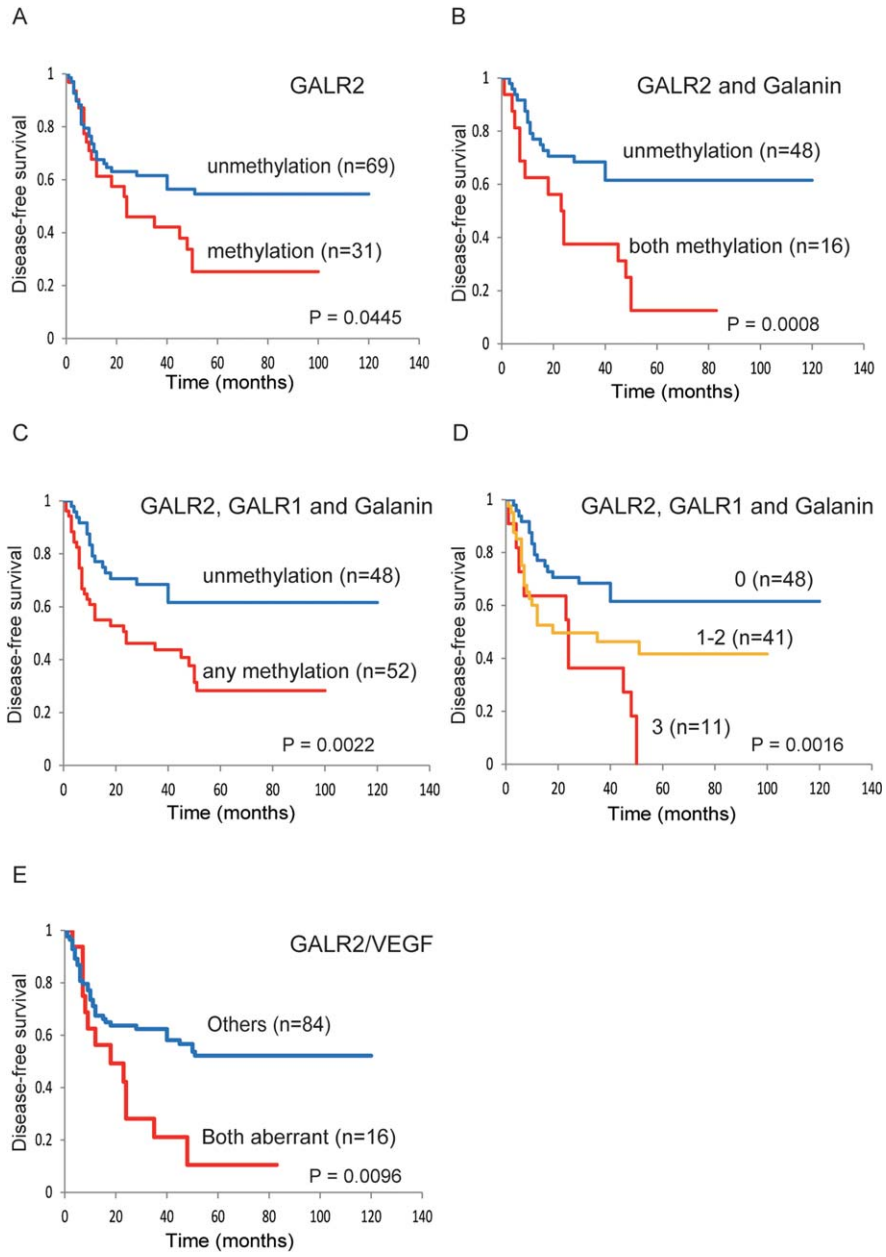

Figure 3. Kaplan-Meier survival curves for patients with head and neck squamous cell carcinoma (HNSCC). Survival time by (A) *GALR2* methylation status; (B) *GALR2* and *Galanin* methylation status; (C) *GALR2*, *GALR1*, and *Galanin* methylation status; (D) number of unfavorable events. Joint analyses of 3 genes showed a significant trend for poorer DFS as the number of unfavorable events increased ($P = .0016$); (E) *GALR2* methylation status and *VEGF* expression status.

rate of 12.5%, as compared with 61.6% in the absence of methylation of these genes (log-rank test, $P = .0008$) (Fig. 3B). Methylation of any 3 genes was associated with a DFS rate of 28.3%, as compared with 61.6% in the absence of methylation of these genes (log-rank test, $P = .0022$) (Fig. 3C). In *GALR2*, *GALR1*, and *Galanin*, the DFS rates for zero methylated genes, 1 to 2 methylated genes, and all 3 methylated genes, were 61.6%, 41.7%, and 0%, respectively (log-rank test, $P = .0016$; Fig. 3D). Multivariate logistic-regression analysis, which involved age, sex, smoking status, alcohol intake, stage grouping and methylated genes, indicated the estimated odds of recurrence associated with methylation of *GALR2* and the other 8 genes. When *GALR2* was methylated in the primary tumor, the adjusted odds ratio for recurrence was 3.12 (95% confidence interval [CI] = 1.16-8.43;

$P = .024$). Patients with methylation of both *Galanin* and *GALR2* had a significantly higher odds ratio for recurrence of 9.05 (95% CI = 1.76-46.5; $P = .008$), compared with those not exhibiting methylation for this pair of genes (Fig. 4A). Multivariate logistic-regression analysis, which involved age, sex, smoking status, alcohol intake, global methylation value, and methylated genes, indicated the estimated odds of recurrence associated with methylation of *GALR2*, *GALR1*, and *Galanin*. *GALR2* methylation is an independent biomarker on its own, and patients with methylation of *GALR2* exhibited a high odds ratio for recurrence of 2.71 (95% CI = 1.01-7.31; $P = .0489$) (Fig. 4B).

Suppression of Cell Growth After Restoration of GALR2 *Expression*

The UM-SCC-1 cell line, which exhibits extensive hypermethylation and expresses no *GALR1* and *GALR2* message, was used for assessment of exogenous *GALR2* reexpression and effects on cell proliferation in response to galanin stimulation. UMSCC-1 cells transfected with wild-type *GALR2* in the pCMVIresGFP or pCMV *GALR2*HAIresGFP constructs were assessed for *GALR2* expression by immunoblotting (Fig. 5A) and immunocytochemistry (Fig. 5B) with HA-tag–specific antibody (Fig. 5A). GALR2-HA expression was clearly detected, and GALR2-HA localized to the cytoplasmic membrane as expected for a GPCR (Fig. 5B). Treatment with 0.5 μM *Galanin* suppressed cell proliferation in UM-SCC-1 *GALR2*HA cells by 33% ($P < .01$) relative to the vector alone in transfected cells (Fig. 5C). Thus, *GALR2* inhibits tumor cell proliferation in response to *Galanin* stimulation.

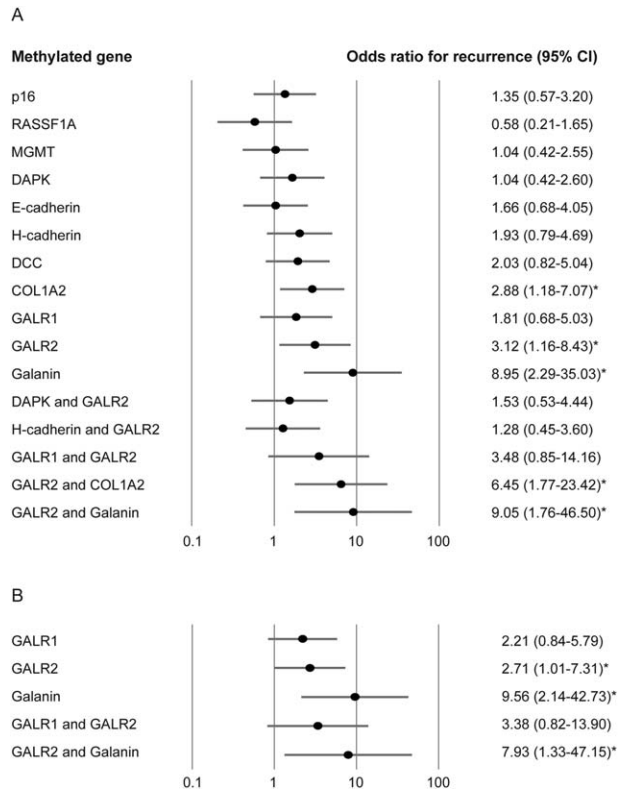

Figure 4. Multivariate logistic-regression model. (A) Odds ratios for recurrence are reported on the basis of the multivariate logistic regression model adjusted for age (70 and older versus < 70), sex, smoking status, alcohol intake, and stage (I, II, III, versus IV). A multivariate logistic regression analysis showed the estimated odds of recurrence to be associated with the methylation of *GALR2* and 8 other genes. (B) Odds ratios for recurrence are reported on the basis of the multivariate logistic regression model adjusted for age (70 years and older versus < 70 years), sex, smoking status, alcohol exposure, and global methylation value (6 to 8 versus < 6). CI = confidence interval.

DISCUSSION

Our preliminary analysis showed that silencing of the *GALR1* gene by methylation may be a critical event in tumor progression of HNSCC, because 18q loss of heterozygosity is associated with tumor progression,[18] and *GALR1* promoter methylation was associated with reduced overall survival rates.[3] Furthermore, the methylation of *Galanin* significantly correlated with *GALR1* methylation and reduced DFS. The methylation of the gene pair *Galanin* and *GALR1* in the primary tumor was associated with the most significant odds ratio of recurrence,[4] whereas another study concluded that *GALR1* induces cell cycle arrest, and *GALR2* induces both cell cycle arrest and apoptosis in HNSCC following *Galanin* treatment.[2,5] Results of this study indicated that aberrant cycling D1 expression and DNA methylation of *GALR2* is associated with prognosis.

In regard to GALRs, another study demonstrated that induction of high levels of *GALR2* expression mediated apoptosis and inhibition of cell proliferation in neuroblastoma cells, but *GALR1*-expressing cells exhibited reduced potency to decrease cell proliferation.[6] Furthermore, the mitogenic effects of *Galanin* on small cell lung carcinoma cells are mediated by a single galanin receptor subtype, *GALR2*.[19] Although GalR2 knockout mice are known to exhibit anxiety- and depression-related behaviors, no data are available indicating that they are susceptible to spontaneous tumor development. Thus, the role of GALR2 in inducing tumorigenesis and the mechanisms involved are not well-established.

Recent advances in molecular biology have made it possible to apply new strategies such as gene- and molecular-targeted therapies as suitable cancer treatments.

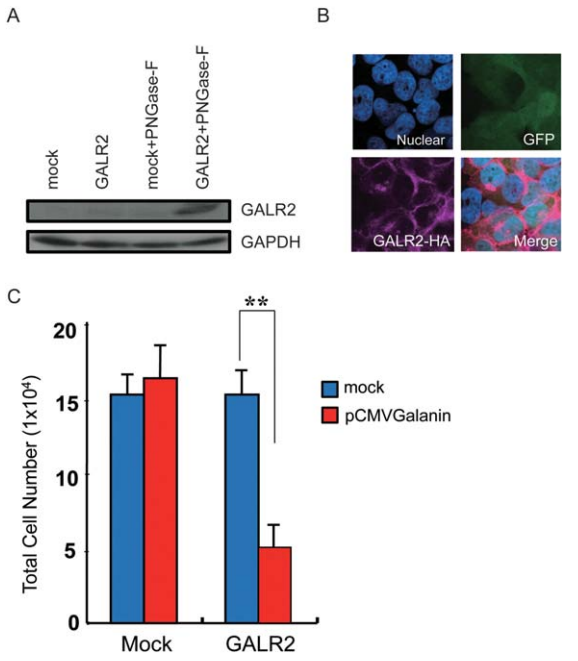

Figure 5. Exogenous *GALR2* expression in transfected UM-SCC-1 cells. (A) Immunoblotting shows exogenous *GALR2* expression in pCMV*GALR2*IresGFP-transfected cells detected using antibody to hemagglutinin (HA)-tag. Lanes labeled mock and *GALR2*HA contain protein lysates from mock-transfected and *GALR2*HA-transfected UM-SCC-1 cells (UM-SCC-1-*GALR2*), respectively. Lanes labeled mock+PNGase-F and *GALR2*HA+PNGase-F contain protein from cell lysates that were digested with N-glycosidase F. (B) Exogenous *GALR2* localizes to the cell membrane in UM-SCC-1-*GALR2* cells. UM-SCC-1-*GALR2* cells were stained with mouse monoclonal anti–HA-tag antibody and Hoechst 33342. Photographs show cells stained with Hoechst 33342 (upper left), green fluorescent protein (GFP; upper right), HA-tag (lower left), and the merged image (lower right); magnification ×400. (C) Relative colony-formation ability for UM-SCC-1. The vector-transfected number of hygromycin B–resistant colonies was set at 100%. Colonies larger than 1 mm were counted, and the results are presented as the mean ± standard deviation (bars) of 3 separate experiments, each performed in triplicate.

Despite these advances, applications for HNSCC have trailed behind innovative treatments for breast, renal, and colorectal carcinomas.[2,5] GPCRs belong to a superfamily of cell surface signaling proteins that play pivotal roles in many physiological functions and in multiple diseases, including the development of cancer and cancer metastasis.[20] In recent years, several studies have suggested that these neuropeptides function as tumor suppressors and possess potent antitumor properties for human cancers. Hypermethylation of *GALR2* has been described in several cancers, including colorectal cancer, breast cancer,[8] and hepatocellular carcinoma.[9] Despite our understanding of adenocarcinoma, hypermethylation of *GALR2* in squamous cell carcinoma (SCC) such as head and neck, esophageal, lung, and cervical cancers, is an area that remains to be investigated. In this regard, it is worth noting that *GALR2* promoter methylation and silencing may occur frequently in HNSCC and this association with *GALR1* methylation, *Galanin* methylation, and decreased survival, is statistically significant.

Recently, *GALR2* was identified as a chemosensitive methylation candidate for bevacizumab regimens used to treat colorectal cancer cells.[21] A previous study from our laboratory showed that high VEGF expression was significantly related to a decrease in survival, whereas loss of VEGF results in a significantly improved prognosis.[14] Furthermore, Yamatodani et al reported significant inhibitions of bevacizumab on angiogenesis and cancer cell survival in HNSCC.[22] Our results indicate that hypermethylation of *GALR2* with a high expression of *VEGF* is associated with a DFS rate of 10.5%, compared with 52.2% for other conditions (log-rank test, $P = .0096$) (Fig. 3E). Because the exact mechanisms of the functions of *GALR2* and *VEGF* genes in tumorigenesis are still unknown, it will be interesting to elucidate the relationships between the *GALR2* and *VEGF* genes. At present, only a limited number of efficient methylation markers of chemosensitivity have been discovered. Good response to chemotherapy has been described for other DNA repair genes such as *WRN* methylation for irinotecan in colorectal tumors[23] and *IGFBP-3* methylation for cisplatin in lung tumors.[24] Recently, methylation of *DAPK* was reported to be associated with cetuximab and erlotinib resistance in non–small cell lung cancer and HNSCC cell lines.[25] Because our study did not include a patient receiving chemotherapy, both *GALR2* methylation and *VEGF* expression should be investigated as potential chemosensitive candidates for targeted therapy in large clinical cohorts.

In this study, *GALR2* expression was frequently absent in HNSCC. The *GALR2* expression-suppressive UM-SCC cell lines were hypermethylated in the CpG islands of the *GALR2* promoter region. Furthermore, we demonstrated that re-expression of *GALR2* in transfected cells results in galanin-induced inhibition of cell proliferation. In 100 primary tumor samples, hypermethylation of the *GALR2* promoter (31%) was also documented in a similar proportion to the hypermethylation of promoters of other genes (24%-58%). Importantly, there is a significant correlation between *GALR2* promoter methylation and *Galanin* methylation status in primary samples. Our preliminary studies have shown that *GALR1* and *Galanin* methylation were also found in 38% and 24% of primary tumor specimens, respectively.[3,4] Similarly, concurrent

analysis revealed that 11.0% of the tumors were completely methylated in all 3 genes; 41.0% exhibited methylation of either *GALR2*, *GALR1*, or *Galanin*; and 48.0% exhibited no methylation in any of the 3 genes. A total of 52.0% of all cases exhibited identical promoter hypermethylation in at least 1 of the 3 genes investigated. Patients who were identified with hypermethylation of both *GALR2* and *Galanin* genes experienced shorter DFS than did patients exhibiting different genetic methylation patterns. In multivariate logistic-regression analyses, methylation of the *GALR2* gene was associated with a significant odds ratios of recurrence. The feasibility of clinically useful epigenetic markers in HNSCC is becoming clearer, not only through this study but also based on results of a recently published study by Carvalho et al.[26] Our findings support the translation of such methylation markers into clinical practice, although additional prospective studies will be required to validate these genes in larger populations of patients with HNSCC.

Thus, *GALR2* resembles other major tumor suppressor genes in terms of frequency of aberrant promoter methylation both in vitro and in vivo. In this study, we showed for the first time, to our knowledge, that expression of *GALR2* mRNA is lost in HNSCC as a consequence of DNA methylation. Furthermore, silencing of the *GALR2* gene by methylation may be a critical event in HNSCC. The current data suggest that *GALR2*, *GALR1*, and *Galanin* are potentially significant therapeutic targets and prognostic factors in HNSCC.

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CONFLICT OF INTEREST DISCLOSURE

The authors made no disclosure.

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