RESEARCH ARTICLE

Aberrant Methylation Inactivates Somatostatin and Somatostatin Receptor Type 1 in Head and Neck Squamous Cell Carcinoma

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Abstract

Purpose
The aim of this study was to define somatostatin (SST) and somatostatin receptor type 1 (SSTR1) methylation profiles for head and neck squamous cell carcinoma (HNSCC) tumors at diagnosis and follow up and to evaluate their prognostic significance and value as a biomarker.

Methods
Gene expression was measured by quantitative RT-PCR. Promoter methylation status was determined by quantitative methylation-specific PCR (Q-MSP) in HNSCC.

Results
Methylation was associated with transcription inhibition. SST methylation in 81% of HNSCC tumor specimens significantly correlated with tumor size (P = 0.043), stage (P = 0.008), galanin receptor type 2 (GALR2) methylation (P = 0.041), and tachykinin-1 (TAC1) (P = 0.040). SSTR1 hypermethylation in 64% of cases was correlated with tumor size (P = 0.037), stage (P = 0.037), SST methylation (P < 0.001), and expression of galanin (P = 0.03), GALR2 (P = 0.014), TAC1 (P = 0.023), and tachykinin receptor type 1 (TACR1) (P = 0.003). SST and SSTR1 promoter hypermethylation showed highly discriminating receiver operator characteristic curve profiles, which clearly distinguished HNSCC from adjacent normal mucosal tissues. Concurrent hypermethylation of galanin and SSTR1 promoters correlated with reduced disease-free survival (log-rank test, P = 0.0001). Among patients with oral cavity and oropharynx cancer, methylation of both SST and SSTR1 promoters correlated with reduced disease-free survival (log-rank test, P = 0.028). In multivariate logistic-regression analysis,
concomitant methylation of galanin and SSTR1 was associated with an odds ratio for recurrence of 12.53 (95% CI, 2.62 to 59.8; \( P = 0.002 \)).

**Conclusions**

CpG hypermethylation is a likely mechanism of SST and SSTR1 gene inactivation, supporting the hypothesis that SST and SSTR1 play a role in the tumorigenesis of HNSCC and that this hypermethylation may serve as an important biomarker.

**Introduction**

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most frequent type of cancer. [1] The use of targeted drugs is an increasingly adopted anticancer strategy; the application of epidermal growth factor receptor (EGFR)-specific antibodies combined with radiotherapy is a prominent example. However, despite high expression of EGFR in HNSCC, EGFR inhibitor monotherapy has only a modest impact on survival. [2] Recently, a tumor suppressor role for neuropeptides that is mediated via the autocrine and/or paracrine systems has been proposed. [3] Our findings suggest that simultaneous methylation of galanin, galanin receptor type 1 (GALR1), and GALR2 genes occurs in a subset of HNSCC and may be used as a prognostic marker. [4,5] Somatostatin (SST) was first identified as a growth hormone release-inhibitory factor in ovine hypothalamus in 1973. [6] Its main functions involve regulating endocrine and exocrine secretion, modulating motor activity, and inhibiting gastrin-stimulated gastric acid secretion in the gastrointestinal tract. [7] In recent years, several studies have suggested that SST functions as a tumor suppressor gene and possesses potent antitumor and antisecretory activities in several human cancers in vitro and in vivo. [7] SST suppresses tumor growth through distinct mechanisms that involve inhibition of growth factors and hormones, reduction in vascularization, and regulation of the immune system. [8] Hypermethylation of SST has been described in esophageal cancer, [7] gastric cancer, [9] colon cancer, [10] and renal cancer. [11] Promoter hypermethylation concomitant with transcriptional silencing of SSTR1 expression has been detected in EBV-positive gastric cancer cells. [12] Despite our understanding of gastrointestinal tract cancer, hypermethylation in head and neck cancer remains to be explored. The purpose of this study was to first define a SST and SSTR1 methylation profile in HNSCC tumors analyzed at the time of diagnosis and then to evaluate its value as a prognostic and recurrence biomarker.

Neuroendocrine peptides play essential roles in the regulation of gastrointestinal endocrine and exocrine secretion, motility, and mucosal immunity. Moreover, some neuroendocrine peptides, including SST, have been implicated in the modulation of human tumorigenesis by both direct and indirect means. The current findings provide novel direct epigenetic evidence in human patients for the involvement of SST in the process of human tumor suppression. [10] Kharmate et al. reported that SSTR1 controls EGF-mediated cell survival via dissociation of an ErbB heteromeric complex. [13] Others recently reported that both SSTRs and ErbBs activate the MAPK pathway, as SST-induced MAPK activation results in delayed cell cycle progression, whereas EGF activation promotes proliferation. [14] Therefore, detection of aberrant expression of SST/SSTR1 may be of potential use as a marker for selecting HNSCC patients who could benefit from additional targeted therapies.

To test this hypothesis, we studied methylation of the SST and SSTR1 promoters by Q-MSP in 100 head and neck tumors of differing primary sites. More recently, data from our
laboratory have shown that the galanin, GALR1, GALR2, TAC1, and TACR1 promoters are methylated in HNSCC. [15,16] Therefore, we hypothesized that neuropeptide genes and receptor genes might be inactivated via promoter hypermethylation in human head and neck cancers, and that hypermethylation of these genes is an important event in the genesis of HNSCC. Moreover, we discovered a unique inverse relationship between SST and SSTR1 promoter hypermethylation and other neuropeptide genes.

Materials and Methods

Tumor samples and cell lines

Tumor specimens in an original cohort were obtained at surgery from 100 primary HNSCC samples. All patients were treated at the Department of Otolaryngology, Hamamatsu University School of Medicine, between 1977 and 1995. Clinical information including age, sex, tumor site, smoking status, alcohol exposure, tumor size, lymph node status, and stage grouping were obtained from the clinical records. The mean age was 63.6 years (range 39–93 years), and the male:female ratio was 78:22. The primary tumor was located in the oral cavity (n = 34), the hypopharynx (n = 24), the larynx (n = 20), the oropharynx (n = 11), and the paranasal cavity (n = 11). Matched pairs of head and neck tumor tissues and adjacent normal mucosal tissues were obtained from the surgical specimens of 36 patients for initial methylation screening between 2008 and 2011. The normal oropharynx samples were obtained from chronic tonsillitis patients after tonsillectomy. All patients provided written informed consent under a protocol approved by the Institutional Review Boards at the Hamamatsu University School of Medicine. DNA and cDNA were derived from 12 UM-SCC cell lines established from patients at the University of Michigan. Fibroblasts from the original tumor specimen were used as the source of normal somatic DNA. [17] Nonmalignant cells from the donors of UM-SCC cell lines have the same number [e.g., UM-SCC-6 and UM-6F (fibroblasts)]. Other control cells included normal human keratinocytes (NHK).[18] 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. [4,5]

Bisulfite modification and quantitative methylation-specific PCR (Q-MSP)

Genomic DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Bisulfite modification of genomic DNA was performed as reported in a previous study. [4] Promoter methylation of SST was measured by quantitative methylation-specific PCR (Q-MSP) with the TaKaRa Thermal Cycler Dice TM Real-Time System TP800 (TaKaRa, Tokyo, Japan). Q-MSP primers for methylated DNA were Q-MSP-SST-F (5’- GGG GCG TTT TTT AGT TTG ACG T-3’) and Q-MSP-SST-R (5’-AAC AAC GAT AAC TCC GAA CCT CG-3’), Q-MSP-SSTR1-F (5’- CGG GTG CGC GAG GAG AAA GTT-3’) and Q-MSP-SSTR1-R (5’- TAG TTC GGG TAG TTG CGG CGA A-3’), and Q-MSP-AC1-F (5’-TGG TGA TGG AGG AAG TTT AGT AAG T-3’) and Q-MSP-AC1-R (5’-AAC CAA TAA AAC CTA CTC CCT TAA-3’). Q-MSP was carried out and the normalized methylation value (NMV) was defined as described previously. [4] To analyze the methylation status of TAC1 [16], TACR1 [16], Galanin [15], GALRI [4] and GALR2 [5] primers, conditions, as described previously, were used.

Quantitative RT-PCR of SST and SSTR1

Total RNA was isolated with the RNasy Mini Kit (QIAGEN, Hilden, Germany) and treated with RNase-Free DNase (QIAGEN). cDNA was generated from DNase-treated total RNA by using random primers (Invitrogen, Carlsbad, CA) with Superscript II reverse transcriptase.
The primer sequences were as follows: SST forward, 5’-CCA GAC TCC GTC AGT TTC TGC A-3’; SST reverse, 5’-CAT CAT TCT CCG TCT GTT TGG GTT-3’ [10]; SST1 forward, 5’- TCT GCG CGA AGA TCG TCA AC-3’; SST1 reverse, 5’- GCG GCT CTG GAC TGG TAA ATG-3’ (TaKaRa, Tokyo, Japan); GAPDH forward, 5’-GCA CCG TCA AGG CTG AGA AC-3’; and GAPDH reverse, 5’-TGG TGA AGA CGCCAG TGG A-3’. To analyze the expression of SSTR2, SSTR3, SSTR4, and SSTR5, we used previously described primers and conditions. [19] Quantitative RT-PCR was performed on the TaKaRa TP800 system.

Statistical analysis

Receiver-operator characteristic (ROC) curves were generated by using the NMVs for the 36 HNSCC and 36 adjacent normal mucosal tissues. The area under the ROC curve identified optimal sensitivity and specificity levels at which normal tissues could be distinguished from HNSCC tissues, and corresponding NMV thresholds were calculated for SST. The cutoff value determined from this ROC curve was applied to determine the frequency of SST methylation.

To determine the overall rate of methylation in individual samples, we used the Methylation Index (MI). [20,21] The MI for each sample was defined as the ratio of the number of methylated genes to the number of genes tested (seven in this study; SST, SSTR1, TAC1, TACR1, Galanin, GALR1 and GALR2). Global Methylation Index (GMI) was calculated by taking the sum of gene promoter hypermethylation events in each tumor divided by the number of genes examined (eight in this study; p16, RASSF1A, E-cadherin, H-cadherin, MGMT, DAPK, DCC, and COL1A2). Mean differences in MI and GMI by histological types and cancer risk factors were examined by employing stratified analysis of variance (Student’s t-test).

For frequency analysis in contingency tables, associations between variables were analyzed by Fisher’s exact test. Comparisons and tests for statistical significance in the colony formation assay were made with the Student’s t-test. The disease-free interval was measured from the date of treatment to the date when locoregional recurrence or distant metastasis was diagnosed. Disease-free survival (DFS) probabilities were estimated by the Kaplan–Meier method, and the log-rank test was applied to assess the significance of differences between actuarial survival curves. Multivariate logistic-regression analysis, which involved age, sex, smoking status, alcohol intake, stage grouping, and methylated genes, was used to identify the predictive value of the prognostic factors. [1,22] P values are two-tailed and significance was determined as $P < 0.05$. All statistical analyses were performed with StatMate IV (ATMS Co. Ltd., Tokyo, Japan).

Results

UM-SCC cell lines

Quantitative RT-PCR of SST1 and SSTR1 transcripts from 10 UM-SCC cell lines revealed lower expression in cancer cell lines than in normal fibroblasts ($P < 0.01$, Fig. 1A, 1B). Q-MSP technology indicated a significantly increased NMV of SST1 promoter methylation in cancer cell lines versus normal fibroblasts and keratinocytes ($P < 0.01$, Fig. 1C). The NMV of SSTR1 was significantly higher in UMSCC than in normal fibroblasts and keratinocytes ($P < 0.05$, Student’s t-test) (Fig. 1D).

Matched pairs of head and neck tumors and adjacent normal mucosal tissues

SST and SSTR1 promoter methylation status were analyzed by Q-MSP in 36 cancerous and paired noncancerous mucosa. SST and SSTR1 methylation levels were significantly higher in
primary HNSCCs than in noncancerous mucosal tissues (median NMV = 0.331 versus 0.021, \( P < 0.001 \), and median NMV = 0.067 versus 0.001, \( P < 0.01 \), Wilcoxon matched-pairs test and paired Student’s \( t \)-test; Fig. 2A, 2B). \( SST \) and \( SSTR1 \) promoter hypermethylation showed highly discriminative ROC curve profiles, which clearly distinguished HNSCC from normal mucosal tissues (AUROC = 0.9375, AUROC = 0.9522, respectively). ROC curves with corresponding areas under the ROC curve for target genes in HNSCC versus normal mucosal tissues is shown in Fig. 2C and 2D. The cutoff NMVs for \( SST \) (0.041) and \( SSTR1 \) (0.012) were chosen from the ROC curves for high sensitivity and > 95% specificity (Fig. 2C, 2D).
Clinicopathologic characteristics of 100 primary HNSCC samples

We classified a specimen as methylated when the NMV exceeded the cutoff value. The SST promoter was methylated in 81 of 100 (81%) cases (Table 1). The SSTR1 promoter was methylated in 64 of 100 (64%) cases (Table 1). There was a significant correlation between the methylation status of the SST and SSTR1 promoters ($P < 0.001$). Methylation of SST was associated with several clinicopathologic factors, including tumor size ($P = 0.043$), stage ($P = 0.008$),

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Fig 2. Hypermethylation patterns in matched pairs of head and neck tumors and adjacent normal mucosal tissues. (A) SST NMVs of head and neck tumors were significantly higher than those of paired adjacent normal mucosal tissues ($P < 0.001$). (B) A higher frequency and quantity of SSTR1 methylation was noted in head and neck tumors than in matched normal mucosal tissues ($P < 0.01$). (C) The area under the ROC curve (AUROC) value for the SST gene was 0.9375. At the cutoff value of 0.046, sensitivity was 80.6% and specificity was 94.4%. (D) The AUROC value for the SSTR1 gene was 0.9522. At the cutoff value of 0.012, sensitivity was 61.1% and specificity was 100%.

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Comparison of MI among selected epidemiologic and clinical characteristics

A representative methylation analysis for SST, SSTR1, TAC1, TACR1, Galanin, GALR1, and GALR2 in tumors is shown in Fig. 3A. Fifty-eight percent (58 of 100) of the tumors included 0 to 3 hypermethylated genes: 17% had 4 hypermethylated genes, 11% had 5 hypermethylated genes, and 14% had 6 or 7 hypermethylated genes (Fig. 3A). We evaluated MI using promoter

### Table 1. SST and SSTR1 Genes Methylation Status in HNSCC Primary Samples.

| Characteristics (n = 100) | SST   | SSTR1  |
|--------------------------|-------|--------|
|                         | Present (81) | Absent (19) | P-value† | Present (64) | Absent (36) | P-value† |
| Age                     | 70 and older (29) | 24 | 5 | 1 | 18 | 11 | 1 |
|                         | Under 70 (71)  | 57 | 14 | 1 | 46 | 25 | 1 |
| Gender                  | Male (78)     | 65 | 13 | 1 | 51 | 27 | 1 |
|                         | Female (22)   | 16 | 6  | 1 | 13 | 9  | 1 |
| Smoking status          | Smoker (66)   | 53 | 13 | 1 | 45 | 21 | 1 |
|                         | Nonsmoker (34) | 28 | 6  | 1 | 19 | 15 | 1 |
| Alcohol exposure        | Ever (60)     | 49 | 11 | 1 | 39 | 21 | 1 |
|                         | Never (40)    | 32 | 8  | 1 | 25 | 15 | 1 |
| Tumor size              | T1–2 (52)     | 38 | 14 | 0.043 | 28 | 24 | 0.037 |
|                         | T3–4 (48)     | 43 | 5  | 1 | 36 | 12 | 1 |
| Lympho-node status      | N0 (46)       | 34 | 12 | 0.126 | 26 | 20 | 0.21 |
|                         | N+ (54)       | 47 | 7  | 1 | 38 | 16 | 1 |
| Stage                   | I–III (49)    | 34 | 15 | 0.008 | 26 | 23 | 0.037 |
|                         | IV (51)       | 47 | 4  | 1 | 38 | 13 | 1 |
| Recurrence events       | Positive (47) | 37 | 10 | 0.618 | 32 | 15 | 0.532 |
|                         | Negative (53) | 44 | 9  | 1 | 32 | 21 | 1 |
| SST methylation         | Yes (81)      | -  | -  | - | 60 | 21 | <0.001 |
|                         | No (19)       | -  | -  | 4 | 15 | 1 |
| Galanin methylation     | Yes (25)      | 23 | 2  | 0.185 | 21 | 4  | 0.030 |
|                         | No (75)       | 58 | 17 | 1 | 43 | 32 | 1 |
| GALR1 methylation       | Yes (38)      | 33 | 5  | 0.3 | 29 | 9  | 0.055 |
|                         | No (62)       | 48 | 14 | 1 | 35 | 27 | 1 |
| GALR2 methylation       | Yes (33)      | 31 | 2  | 0.041 | 27 | 6  | 0.014 |
|                         | No (67)       | 50 | 17 | 1 | 37 | 30 | 1 |
| TAC1 methylation        | Yes (49)      | 44 | 5  | 0.040 | 37 | 12 | 0.023 |
|                         | No (51)       | 37 | 14 | 1 | 27 | 24 | 1 |
| TACR1 methylation       | Yes (34)      | 30 | 4  | 0.186 | 29 | 5  | 0.003 |
|                         | No (66)       | 51 | 15 | 1 | 35 | 31 | 1 |

†Fisher’s exact probability test

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GALR2 methylation ($P = 0.041$), TAC1 methylation ($P = 0.040$), and DAPK methylation ($P = 0.012$) (Table 1 and S1 Table). SSTR1 methylation was significantly correlated with tumor size ($P = 0.037$), stage ($P = 0.037$), galanin ($P = 0.030$), GALR2 methylation ($P = 0.014$), TAC1 methylation ($P = 0.023$), TACIR methylation ($P = 0.003$), H-cadherin methylation ($P = 0.007$), MGMT methylation ($P = 0.001$), DAPK methylation ($P = 0.001$) and DCC methylation ($P = 0.045$) (Table 1 and S1 Table).
Fig 3. Gene promoter methylation analysis by quantitative-MSP in 100 primary HNSCC samples. (A) Percentage of patients with epigenetic alternation in SST, SSTR1, TAC1, TACR1, galanin, GALR1, and GALR2 genes. (B) Comparison of MI among selected epidemiologic and clinical characteristics.

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methylation of SST, SSTR1, TAC1, TACR1, Galanin, GALR1, and GALR2. Hypermethylation of MI was significantly associated with tumor size \((P = 0.022)\), lymph-node status \((P = 0.019)\), stage \((P = 0.004)\), and recurrence events \((P = 0.036)\). No differences were noted with regard to age at onset, gender, alcohol exposure, or smoking status (Fig. 3B). Based on data from continuous marker methylation analyses, GMI of p16, RASSF1A, E-cadherin, H-cadherin, MGMT, DAPK, DCC, and COL1A2 were not correlated with any of the characteristics (S1 Fig.).

**Prognostic Value of the SST, SSTR1, and Other Genes**

Kaplan-Meier plots indicated that methylation of SST, SSTR1, and other genes in the patients’ tumors were related to the duration of DFS. SST methylation (log-rank test, \(P = 0.514\)) and SSTR1 methylation (log-rank test, \(P = 0.136\)) were not associated with any difference in DFS (Fig. 4A, 4B). Methylation of both SST and SSTR1 was not associated with an altered DFS rate when compared with samples harboring low levels of methylation (41.7% versus 53.3%, log-rank test, \(P = 0.565\)) (Fig. 4C). Among patients with oral cavity and oropharynx cancer, the disease-free survival rate in the group of patients with both SST and SSTR1 methylation was 48.1% as compared with 81.4% in the other groups (log-rank test, \(P = 0.028\)) (Fig. 4D). The DFS was lower in the MI (4–7) methylated genes group than in the MI (0–3) methylated genes group (14.0% versus 64.7%, respectively; log-rank test, \(P < 0.001\)) (Fig. 4E). The DFS of the both TAC1 and SSTR1 methylation group was significantly higher than that of the no methylation group (log-rank test, \(P = 0.011\)) (S2A Fig.). Methylation of both galanin and SSTR1 was associated with a DFS rate of 0% versus 59.0% in the absence of methylation (log-rank test, \(P = 0.0001\)) (S2B Fig.). Patients in which GALR2 and SSTR1 were not methylated survived significantly longer than those in which both genes were methylated (log-rank test, \(P = 0.005\)) (S2C Fig.). The DFS of the both SSTR1 and GALR1 methylation group was significantly higher than that of the no methylation group (log-rank test, \(P = 0.022\)) (S2D Fig.).

Multivariate logistic-regression analysis showed the estimated odds of recurrence associated with methylation of SST, SSTR1, and other genes. Methylation of either SST or SSTR1 was associated with an elevation in the odds of recurrence that was not significant. Patients with SSTR1 and galanin methylation had a highest odds ratio for recurrence (OR = 12.53, 95% CI, 2.62 to 59.81; \(P = 0.002\)) (Fig. 5). When MI was tested in the multivariate logistic-regression analysis, MI (0–3 vs. 4–7) was independently predictive of DFS after adjustment for stage (Fig. 5) and GMI (S3 Fig.).

**Discussion**

Recent advances in molecular biology have made it possible to apply new strategies, such as gene therapy and molecular targeted therapy for cancer treatment. [23] However, in comparison to lesions such as breast, renal and colorectal carcinoma, HNSCC treatments are less advanced. [24] GPCRs belong to a superfamily of cell surface signaling proteins with a pivotal role in many physiological functions and multiple diseases, including cancer development and metastasis. [25] In recent years, SST and the somatostatin receptor (which belongs to the GPCR family) have been identified as tumor suppressor genes that possess potent antitumor and antisecretory activities in several human cancers in vitro and in vivo. [8] Head and neck tumor specimens expressed SSTR1, SSTR2, SSTR4, and SSTR5, whereas SSTR3 mRNA expression was low. [26] A similar loss of SSTR1 and SSTR2 protein in normal versus malignant tissue has been observed in laryngeal lesions. [27] In our tumor cell series and normal oropharynx samples (NOS), the profiles of somatostatin and somatostatin receptor mRNAs were consistent with those reported in previous studies (S4 Fig.).
Consistent with this, a recent study showed that SST promoter hypermethylation is common in human esophageal adenocarcinoma, gastric cancer, and colon cancer. [7,9,10] Zhao et al. reported that ectopic expression of SSTR1 in gastric cancer cell lines, which exhibit
| Methylated gene       | Odds ratio for recurrence (95% CI) |
|-----------------------|------------------------------------|
| SST                   | 0.63 (0.20-1.87)                   |
| SSTR1                 | 1.63 (0.67-3.94)                   |
| Galanin               | 8.95 (2.29-35.0)*                  |
| GALR1                 | 1.84 (0.68-5.03)                   |
| GALR2                 | 3.12 (1.16-8.43)*                  |
| TAC1                  | 3.35 (1.37-8.19)*                  |
| TACR1                 | 1.12 (0.45-2.81)                   |
| SST and SSTR1         | 1.16 (0.49-2.70)                   |
| SST and Galanin       | 8.77 (2.28-33.7)*                  |
| SSTR1 and Galanin     | 12.53 (2.62-59.8)*                 |
| SST and GALR1         | 1.31 (0.49-3.47)                   |
| SSTR1 and GALR1       | 1.64 (0.61-4.47)                   |
| SST and GALR2         | 3.02 (1.16-7.81)*                  |
| SSTR1 and GALR2       | 6.33 (2.05-19.5)*                  |
| SST and TAC1          | 2.44 (1.01-5.91)*                  |
| SSTR1 and TAC1        | 3.79 (1.48-9.68)*                  |
| SST and TACR1         | 1.01 (0.39-2.60)                   |
| SSTR1 and TACR1       | 1.25 (0.48-3.21)                   |
| MI (0-3 vs. 4-7)      | 4.84 (1.84-12.7)*                  |

Fig 5. Odds ratios for recurrence based on multivariate logistic-regression adjusted for stage (I, II, III vs. IV), age (65 and older vs. <65), sex, alcohol exposure, and smoking status. Methylation of SSTR1 and galanin in the primary tumor was associated with the most significant odds ratios of recurrence.

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hypermethylation and express no SSTR1 mRNA, significantly suppressed cell growth in culture conditions and reduced tumor size in nude mice. Furthermore, methylation-dependent regulation of SST and SSTR1 expression is observed in the chick embryonic liver during the developmental stages. These findings provide a foundation for further studies on the role of neuropeptide genes and their receptors in carcinogenesis, and their potential utility as biomarkers for many types of tumors. Hypermethylation of TAC1 has been described in esophageal cancer, gastric cancer, colon cancer, and breast cancer. Overall patient survival is correlated with TAC1 methylation status in esophageal squamous cell carcinoma, but not in esophageal adenocarcinoma. Our preliminary analysis showed that silencing of
the \textit{TAC1} gene by methylation may be a critical event in tumor progression of HNSCC and that \textit{TAC1} promoter methylation was associated with reduced overall survival rates.\cite{16} Furthermore, the methylation of galanin significantly correlated with \textit{GALR1} and \textit{GALR2} methylation and reduced DFS. \cite{4,5,15} The methylation of the gene pair of galanin and \textit{GALR1} in the primary tumor was associated with the most significant odds ratio of recurrence, \cite{15} while another study concluded that \textit{GALR1} induces cell cycle arrest, and \textit{GALR2} induces both cell cycle arrest and apoptosis in HNSCC following galanin treatment. \cite{24} This study shows that aberrant DNA methylation of \textit{SST} and \textit{SSTR1} is not associated with prognosis.

Certain combinations of DNA hypermethylation patterns can be used as highly sensitive biomarkers, and can be used to identify cancer cells or to predict tumor progression in prostate and lung cancers, respectively. \cite{32} \cite{33} The methylation status of glutathione S-transferase pi 1 (\textit{GSTP1}) was strongly associated with disease outcome in men with suspected prostate cancer. \cite{34} Broch et al. reported that methylation of \textit{p16} and \textit{H-cadherin} was associated with early recurrence of stage I non-small cell lung cancer. \cite{33} However, gene silencing via hypermethylation is still a relatively important idea in the development of HNSCC, and little is known about the contribution of epigenetics to disease progression in HNSCC.

We systematically investigated \textit{SST} and \textit{SSTR1} promoter hypermethylation in primary HNSCC. To our knowledge, neither expression nor promoter hypermethylation of \textit{SST} and \textit{SSTR1} in HNSCC has been reported previously. Our results show that \textit{SST} and \textit{SSTR1} promoter hypermethylation occurs frequently in UM-SCC cell lines and primary tumors. The frequency of \textit{SST} and \textit{SSTR1} hypermethylation was extremely low in normal fibroblasts and keratinocytes and mucosal tissues. ROC curve analysis revealed that the AUROC values of \textit{SST} and \textit{SSTR1} methylation levels were significantly higher in the HNSCC patients. \textit{SST} and \textit{SSTR1} DNA methylation is a potential biomarker that could facilitate the differential diagnosis of HNSCC. An MSP survey of 100 tumor tissue samples demonstrated that hypermethylation of the \textit{SST} promoter (81\%) and \textit{SSTR1} promoter (64\%) occurred with a high frequency. Indeed, these rates were higher than methylation frequencies of other tumor suppressor loci such as \textit{p16} (52\%), \textit{COL1A2} (48\%), \textit{H-cadherin} (43\%) and \textit{E-cadherin} (40\%). A concurrent analysis showed that \textit{SST} and \textit{SSTR1} were completely methylated in 60 (60\%) cases; another 25 (25\%) cases were methylated at either \textit{SST} or \textit{SSTR1}, meaning that 85 (85\%) cases were methylated at one or both \textit{SST} and \textit{SSTR1} promoters. Patients who had hypermethylation of both genes had lower survival rates than did patients without methylation of both genes; however, this was not statistically significant.

In multivariate logistic-regression analyses, adjusted for stage, age, sex, alcohol exposure, and smoking status, methylation of both \textit{SST} and \textit{SSTR1} was associated with an elevation in the odds of recurrence that was not significant. When both \textit{SSTR1} and galanin were methylated in the primary tumors, the adjusted odds ratio for recurrence was higher than in tumors with other methylation patterns at these two loci. In another model of logistic regression with GMI, age, sex, alcohol exposure, and smoking status, \textit{SSTR1} and galanin methylation has the highest odds ratio as an independent biomarker on its own.

Our study indicates that methylation of the promoter regions of neuropeptide genes in a resected HNSCC specimen is associated with tumor recurrence. The current method used to assess risk recurrence in patients with HNSCC is imprecise—indeed, half of such tumors recur after curative surgery. This information can be used to identify patients with high-risk HNSCC who may benefit from adjuvant therapy and close follow up observation after primary tumor resection. Our findings support the translation of such methylation markers into clinical practice, although additional prospective studies are required to validate these genes in larger populations of HNSCC patients.
Supporting Information

S1 Fig. Comparison of GMI among selected epidemiologic and clinical characteristics. GMI was calculated by taking the sum of gene promoter hypermethylation events (eight in this study; \( \text{p16, RASSF1A, E-cadherin, H-cadherin, MGMT, DAPK, DCC, and COL1A2} \)).

S2 Fig. Kaplan-Meier survival curves for patients with HNSCC. Disease-free survival by (A) TAC1 and SSTR1 methylation status, (B) galanin and SSTR1 methylation status, (C) GALR2 and SSTR1 methylation status, and (D) GALR1 and SSTR1 methylation status. Blue line, patients without methylation; red line, patients with methylation.

S3 Fig. Odds ratios for recurrence based on multivariate logistic-regression adjusted for GMI (6–8 vs. <6), age (65 and older vs. <65), sex, alcohol exposure, and smoking status.

S4 Fig. Diagrammatic representation of SST and SSTRs expression analysis by quantitative-RT-PCR in UM-SCC cell lines and normal oropharyngeal samples (NOS).

S1 Table. SST and SSTR1 genes methylation status in HNSCC primary samples. This is correlation with p16, RASSF1A, E-cadherin, H-cadherin, MGMT, DAPK, DCC, and COL1A2 methylation status.

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Author Contributions
Conceived and designed the experiments: KM. Performed the experiments: KM HK DM AI. Analyzed the data: KM YM. Contributed reagents/materials/analysis tools: KM HF TU TK. Wrote the paper: KM HM.

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