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Research Paper

Genes encoding neuropeptide receptors are epigenetic markers in patients with head and neck cancer: a site-specific analysis

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ABSTRACT

Staging and pathological grading systems are useful but imperfect predictors of recurrence in head and neck squamous cell carcinoma (HNSCC). To identify potential prognostic markers, we examined the methylation status of eight neuropeptide receptor gene promoters in 231 head and neck squamous cell carcinomas. The *NPFFR1*, *NPFFR2*, *HCRTR1*, *HCRTR2*, *NPY1R*, *NPY2R*, *NPY4R*, and *NPY5R* promoters were methylated in 80.5%, 79.2%, 67.1%, 73.2%, 35.1%, 36.4%, 38.5%, and 35.9% of the samples, respectively. In a multivariate Cox proportional hazards analysis, the odds ratio for recurrence was 2.044 (95% confidence interval [CI], 1.323–3.156; P = 0.001) when the *NPY2R* promoter was methylated. In patients without lymph node metastasis (n = 100), methylation of *NPY2R* (compared with methylation of the other seven genes) best correlated with poor disease-free survival (DFS) (odds ratio, 2.492; 95% CI, 1.190–5.215; P = 0.015). In patients with oral cancer (n = 69), methylated *NPY1R* and *NPY2R* were independent prognostic factors for poor DFS, both individually and, even more so, in combination (odds ratio, 3.90; 95% CI, 1.523–9.991; P = 0.005). Similar findings were observed for *NPY2R* and *NPY4R* in patients with oropharyngeal cancer (n = 162) (odds ratio, 5.663; 95% CI, 1.507–21.28; P = 0.010).

INTRODUCTION

Head and neck cancer is the eighth most common cancer worldwide with approximately 650,000 new cases reported annually [1]. More than 90% of head and neck cancers are squamous cell carcinomas; hence, the term "head and neck cancer" is often applied to all carcinomas that arise from the epithelium lining the sinonasal tract, oral cavity, pharynx, or larynx and show microscopic evidence of squamous differentiation [2]. The present standard management strategies include constructive and multimodal treatments such as surgery, radiotherapy, and chemotherapy. Despite these aggressive treatments, long-term survival rates are poor and remain between 40% and 50% [3]. Improvement of patient care requires molecular classification of head and neck squamous cell carcinomas (HNSCCs) that provides both prognostic and mechanistic information.

Technological advances revealed aberrant expression of G protein-coupled receptors (GPCRs) in human tumors owing to events such as gene point mutations, gene silencing via promoter methylation, and changes in gene copy number [4]. The GPCR family consists of six receptor groups with different pharmacological properties: rhodopsin-like GPCRs (Class A), secretin-like GPCRs (Class B), metabotropic glutamate receptors (Class C),

fungal mating pheromone receptors (Class D), cAMP receptors (Class E), and frizzled/smoothened receptors (Class F) [5]. Class A, the largest and best-studied group, consists of four subgroups (α, β, γ, and δ) and includes several members that play a major part in tumor biology [6]. Although GPCRs regulate many aspects of tumorigenesis, only a few GPCR inhibitors are currently used to treat cancer. Potential targets for drug development include novel cancer-associated GPCRs identified via genome-wide analyses of several human tumor types [4].

Aberrant promoter methylation, a hallmark of cancer cells, accounts for the inactivation of many tumor suppressor genes. In HNSCC, methylation of gene promoters is a common mechanism of transcriptional silencing [7–9]. Notably, epigenetic repression of GPCR expression correlates with poor prognosis and the response to radiotherapy and chemotherapy [10].

The aim of this study was to determine the methylation status of eight GPCR-encoding genes in HNSCCs and its relationship to recurrence, survival, and clinical characteristics (e.g., tumor location and lymph node metastasis). All eight genes (*NPFFR1*, *NPFFR2*, *HCRTR1*, *HCRTR2*, *NPY1R*, *NPY2R*, *NPY4R*, and *NPY5R*) encode neuropeptide receptors and are in the Class A β subgroup. This study is the first to implicate neuropeptide receptors in the genesis of HNSCC.

RESULTS

Analysis of the methylation status and expression of neuropeptide receptor genes

Quantitative methylation-specific polymerase chain reaction (PCR) was used to assess the promoter methylation status of eight genes encoding neuropeptide receptors in 231 primary HNSCC samples. At least one of these genes was methylated in almost all samples (229 of 231 samples, 99.1%). The mean number of methylated genes per sample was 4.46 (range, 0–8) (Figure 1A). The methylation rates for the eight genes were as follows: *NPFFR1*, 80.5%; *NPFFR2*, 79.2%; *HCRTR1*, 67.1%; *HCRTR2*, 73.2%; *NPY1R*, 35.1%; *NPY2R*, 36.4%; *NPY4R*, 38.5%; and *NPY5R*, 35.9% (Figure 1B, Supplementary Figure 1). Relative mRNA expression of the eight genes was assessed in 41 of the 231 tumor specimens via quantitative reverse transcription PCR (Supplementary Figure 2).

Correlation between the methylation status of neuropeptide receptor gene promoters and clinicopathological parameters

The methylation index (MI) was defined as the ratio of the number of methylated genes and the number of tested genes in each sample. Continuous marker methylation analyses showed no association between the MI for any of the eight target genes and age at disease onset, sex, alcohol consumption, smoking status, tumor size, lymph node status, clinical stage, or recurrence (Figure 1C).

Associations between the methylation status of the target genes and the clinicopathological features of the patients are summarized in Table 1. Methylation of the *HCRTR2* promoter significantly correlated with lymph node metastasis (P = 0.040), methylation of the *NPY1R* promoter significantly correlated with smoking status (P = 0.041), and methylation of the *NPY2R* promoter significantly correlated with age (P = 0.040) and recurrence (P = 0.004).

Kaplan-Meier analysis

The Kaplan-Meier survival curves for each of the eight target genes in all patients are shown in Figure 2. Disease-free survival (DFS) time did not differ significantly in patients with methylated versus unmethylated genes, with two notable exceptions: it was significantly shorter when *HCRTR2* was unmethylated (P = 0.016) and when *NPY2R* was methylated (P = 0.001). Additional analysis of only the patients without lymph node metastasis (n = 100) revealed shorter DFS times for methylated versus unmethylated *NPY2R* (P = 0.026), but no differences for the other seven genes (Supplementary Figure 3).

Prognostic value of the methylation status of neuropeptide receptor gene promoters

The association between methylation and risk of recurrence was estimated via multivariate analysis using a Cox proportional hazards model adjusted for age, sex, smoking status, alcohol consumption, and clinical stage. In patients in whom the *NPY2R* promoter was methylated (n = 84), the adjusted odds ratio for recurrence was 2.044 (95% confidence interval [CI], 1.323–3.156; P = 0.001) (Table 2). Notably, the odds ratio was significantly higher in patients with no lymph node metastasis (n = 100) in whom the *NPY2R* promoter was methylated versus unmethylated (odds ratio, 2.492; 95% CI, 1.190–5.215; P = 0.015) (Table 3). There was no association between the methylation status of the *HCRTR2* promoter and recurrence regardless of lymph node status (Tables 2, 3). Remarkably, we found that the mode of therapy, HPV status, smoking status, alcohol intake, tumor stage, and gene methylation status all indicated the likelihood of recurrence in patients with methylated *NPY1R* and *NPY2R* promoters. We have included these data in the multivariate analysis in Supplementary Table 1.

Odds ratios for recurrence according to tumor origin were also determined. When the *NPY1R* and*NPY2R* promoters were methylated in patients with oral cancers, the ratios were 2.39 (95% CI, 1.06–5.37; P = 0.036) and 2.93 (95% CI, 1.25–6.88; P = 0.014), respectively, and

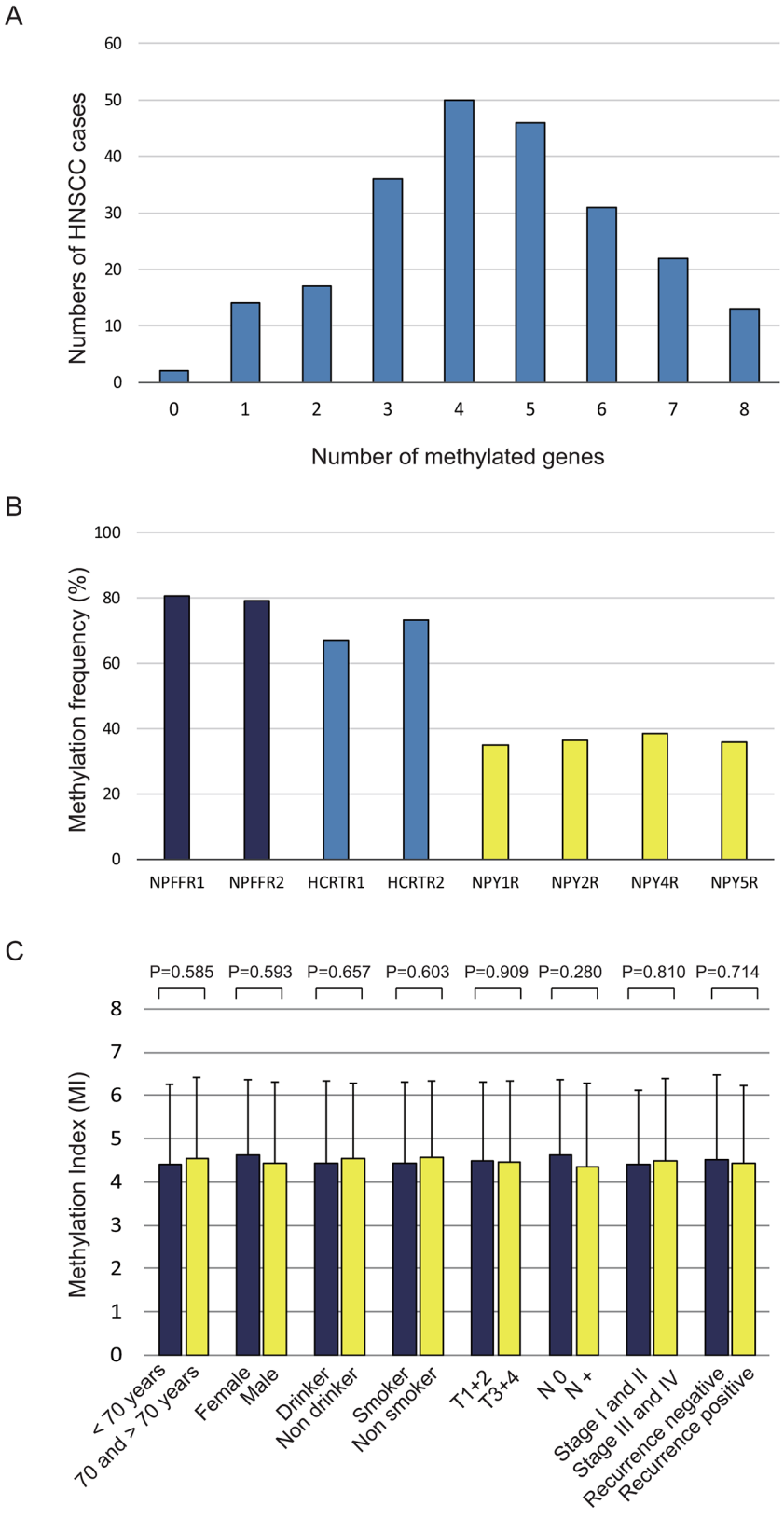

Figure 1: Methylation of the neuropeptide receptor gene promoters in 231 head and neck squamous cell carcinoma samples. **(A)** Bar graph showing the percentage of tumors that express zero to eight of the methylated target genes. **(B)** Bar graph showing the methylation frequencies of the eight genes. **(C)** Bar graph showing the methylation indices (MIs) according to selected clinical parameters. The mean MI for each parameter was determined by using Student's t-test.

Table 1: Distribution of methylation status by selected epidemiologic and clinical characteristics

		Characteristics	Age			Gender			Smoking status			Alcohol exposure		
Gene	Methylation status	Overall(%)	< 70	> 70	P †	Female	Male	P †	Smoker	Non smoker	P †	Drinker	Non drinker	P †
NPFFR1	Yes	186(80.5)	119	67		29	157		140	46		136	50	
	No	45(19.5)	30	15	0.735	7	38	0.995	35	10	0.725	33	12	0.977
NPFFR2	Yes	183(79.2)	118	65		31	152		135	48		134	49	
	No	48(20.8)	31	17	0.989	5	43	0.267	40	8	0.169	35	13	0.966
HCRTR1	Yes	155(67.1)	103	52		26	129		120	35		114	41	
	No	76(33.9)	46	30	0.377	10	66	0.476	55	21	0.4	55	21	0.849
HCRTR2	Yes	169(73.2)	108	61		27	142		126	43		120	49	
	No	62(27.8)	41	21	0.754	9	53	0.786	49	13	0.482	49	13	0.223
NPY1R	Yes	81(35.1)	48	33		14	67		55	26		25	56	
	No	150(64.9)	101	49	0.221	22	128	0.601	120	30	0.041*	37	113	0.31
NPY2R	Yes	84(36.4)	47	37		13	71		64	20		61	23	
	No	147(63.6)	102	45	0.04*	23	124	0.973	111	36	0.908	108	39	0.888
NPY4R	Yes	89(38.5)	55	34		12	77		73	16		70	19	
	No	142(61.5)	94	48	0.496	24	118	0.486	102	40	0.079	99	43	0.136
NPY5R	Yes	83(35.9)	59	24		14	69		61	22		57	26	
	No	148(64.1)	90	58	0.117	22	126	0.687	114	34	0.548	112	36	0.249

	Tumor size			Lympho-node status			Stage			HPV status			Recurrence events		
Gene	T1-2	T3-4	P †	N0	N+	P †	I, II	III, IV	P †	positive	negative	P †	positive	negative	P †
NPFFR1	89	97		83	103		50	136		32	154		63	123	
	23	22	0.694	17	28	0.406	11	34	0.739	5	38	0.492	22	23	0.061
NPFFR2	91	92		82	101		51	132		29	154		67	116	
	21	27	0.461	18	30	0.363	10	38	0.325	8	38	1	18	30	0.91
HCRTR1	73	82		71	84		40	115		26	129		57	98	
	39	37	0.547	29	47	0.27	21	55	0.767	11	63	0.848	28	48	0.992
HCRTR2	85	84		80	89		48	121		27	142		56	113	
	27	35	0.363	20	42	0.04*	13	49	0.256	10	50	1	29	33	0.057
NPY1R	40	41		36	45		18	63		22	59		35	46	
	72	78	0.841	64	86	0.795	43	107	0.289	15	133	0.001*	50	100	0.137
NPY2R	36	48		38	46		20	64		13	71		41	43	
	76	71	0.196	62	85	0.651	41	106	0.498	24	121	1	44	103	0.004*
NPY4R	43	46		37	52		19	70		14	75		32	57	
	69	73	0.967	63	79	0.677	42	100	0.167	23	117	1	53	89	0.834
NPY5R	44	39		34	49		23	60		14	67		33	50	
	68	80	0.303	66	82	0.593	38	110	0.736	23	125	1	52	96	0.484

† Chi-squared test.

* P<0.05.

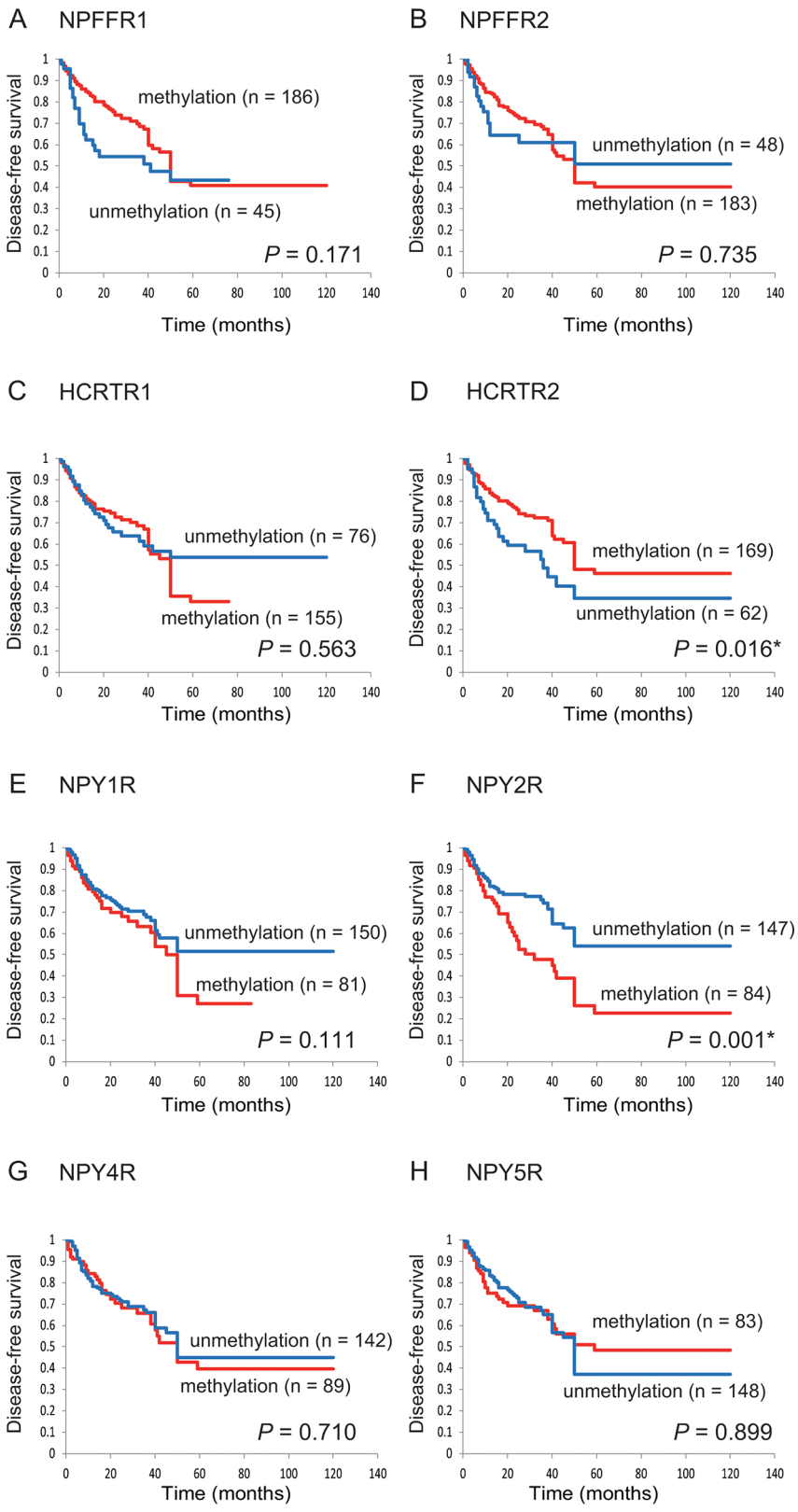

Figure 2: Kaplan-Meier survival curves for the 231 patients with head and neck squamous cell carcinoma according to the methylation status of the eight target genes. Disease-free survival for **(A)** NPFFR1, **(B)** NPFFR2, **(C)** HCRTR1, **(D)** HCRTR2, **(E)** NPY1R, **(F)** NPY2R, **(G)** NPY4R and **(H)** NPY5R. The log-rank test was used to compare the survival times in patients with methylated (red lines) and unmethylated (blue lines) genes.

Table 2: Methylation status of individual genes and associations with disease-free survival using Cox proportional hazards model in 231 patients

Gene	Methylation status	Overall (%)	Recurrence events		Adjusted HR (95% CI) †
			Positive (N = 88)	Negative (N = 143)	
NPFFR1	Yes	186(80.5)	66	120	
	No	45(19.5)	22	23	0.693 (0.424-1.133)
NPFFR2	Yes	183(79.2)	69	114	
	No	48(20.8)	19	29	0.915 (0.540-1.550)
HCRTR1	Yes	155(67.1)	58	97	
	No	76(33.9)	30	46	1.126 (0.708-1.791)
HCRTR2	Yes	169(73.2)	55	114	
	No	62(27.8)	33	29	0.582 (0.320-1.059)
NPY1R	Yes	81(35.1)	32	49	
	No	150(64.9)	56	94	1.407 (0.910-2.176)
NPY2R	Yes	84(36.4)	42	42	
	No	147(63.6)	46	101	2.044 (1.323-3.156)*
NPY4R	Yes	89(38.5)	33	56	
	No	142(61.5)	55	87	1.033 (0.660-1.617)
NPY5R	Yes	83(35.9)	32	51	
	No	148(64.1)	56	92	1.006 (0.646-1.567)

† Adjusted for age, gender, smoking status, alcohol exposure and stage.

* $P<0.05$.

the hazard rate was 3.9 times higher (95% CI, 1.52–9.99; P = 0.005) (Figure 3). Methylation of the *NPY2R* and *NPY4R* promoters correlated positively with recurrence in patients with oropharyngeal cancers, both individually (odds ratio, 5.20; 95% CI, 1.67–16.2; P = 0.005 and odds ratio, 4.90; 95% CI, 1.07–22.4; P = 0.004, respectively) and together (odds ratio, 5.66; 95 % CI, 1.51–21.3; P = 0.010).

External validation of our results using data from The Cancer Genome Atlas (TCGA) database

The methylation status of neuropeptide receptor gene promoters was determined in an additional 516 HNSCC samples and 50 normal samples. The average β values for promoter methylation were significantly higher in the HNSCC samples than in the normal samples (P < 0.005) (Supplementary Figure 4). mRNA expression data were available for 497 HNSCC samples and 20 normal samples. *NPFFR2, HCRTR2, NPY1R, NPY2R*, and *NPY5R* promoter methylation correlated inversely with their respective mRNA levels in both the HNSCC and normal tissue samples (Supplementary Figure 5). To validate the prognostic implications of neuropeptide receptor gene methylation, we examined the data for the 386 HNSCC patients in TCGA database. DFS time was significantly longer in patients with an unmethylated *HCRTR1* promoter than in those with a methylated *HCRTR1* promoter (P = 0.038) (Supplementary Figure 6).

DISCUSSION

Identifying epigenetic modifications of the *NPFFR*, *HCRTR*, and *NPYR* genes is important for understanding how tumors arise and whether they will recur. Using real-time PCR, we examined the methylation status of these genes, all of which encode G protein-coupled neuropeptide receptors, in 231 HNSCCs originating in the hypopharynx, larynx, oropharynx, or oral cavity. We also compared the methylation status of matched HNSCC and normal samples using data from TCGA database. We found that aberrant methylation of the *NPY1R*, *NPY2R*, and *NPY4R* promoters correlated positively with recurrence in patients with HNSCC.

Members of the GPCR family include the neuropeptide FF (NPFF) receptors, of which there are two subtypes, NPFFR1 and NPFFR2, which bind the RF-amide related peptides and FF neuropeptides, respectively [11]. NPFFR1 and NPFFR2 are about 50% identical and are closely related to neuropeptide Y receptors and orexin

Table 3: Methylation status of individual genes and associations with disease-free survival using Cox proportional hazards model in 100 patients with N0 lymph-node status

Gene	Methylation status	Overall (%)	Recurrence events		Adjusted HR (95% CI) †
			Positive (N = 30)	Negative (N = 70)	
NPFFR1	Yes	83(83.0)	23	60	
	No	17(17.0)	7	10	0.831 (0.348-1.981)
NPFFR2	Yes	82(82.0)	23	59	
	No	18(18.0)	7	11	0.656 (0.266-1.617)
HCRTR1	Yes	71(71.0)	22	49	
	No	29(29.0)	8	21	1.162 (0.469-2.879)
HCRTR2	Yes	80(80.0)	22	58	
	No	20(20.0)	8	12	0.465 (0.192-1.131)
NPY1R	Yes	36(36.0)	14	22	
	No	64(64.0)	16	48	1.568 (0.736-3.342)
NPY2R	Yes	38(38.0)	16	22	
	No	62(62.0)	14	48	2.492 (1.190-5.215)*
NPY4R	Yes	37(37.0)	12	25	
	No	63(63.0)	18	45	0.926 (0.423-2.030)
NPY5R	Yes	34(34.0)	12	22	
	No	66(66.0)	18	48	1.118 (0.523-2.387)

† Adjusted for age, gender, smoking status, alcohol exposure and stage.

* $P<0.05$.

receptors (30–35% homology) [12]. Classically, the actions of orexins are mediated by two receptors, orexin receptor type 1 and type 2, which are encoded by the *HCRTR1* and *HCRTR2* genes, respectively. Orexins transiently increase intracellular calcium levels through Gq-dependent and -independent pathways [13] and markedly inhibit cell proliferation in various cancer cell lines by inducing apoptosis [14]. In a previous study, primary colorectal tumors and hepatic metastases expressed *HCRTR1* mRNA regardless of their location or Dukes stage, whereas adjacent normal colonocytes did not express *HCTRTR1* mRNA [15]. Loss of expression of *HCRTR2* correlates with hypermethylation of *HCRTR2* in endometrial cancer compared with normal endometrium [16].

Neuropeptide Y (NPY) activates five GPCRs, namely, NPY1R, NPY2R, NPY4R, NPY5R, and NPY6R [17]. It is one of the most abundantly distributed neurotransmitters and vasoconstrictors in the central and peripheral nervous system. Although the *NPY6R* gene is functional in rabbits and mice, it is absent in rats and considered a pseudogene in primates and pigs [18]. NPY regulates food intake, blood pressure, and circadian rhythms, as well as other physiological activities [19]. Via NPY1R, NPY inhibits the growth of hepatocellular carcinomas by inactivating the mitogen-activated protein kinase signaling pathway [20]. Significant associations between cumulative arsenic exposure and the methylation level of the *NPY2R* gene have been observed in smoking-unrelated urothelial carcinomas [21]. NPY2R is often strongly expressed in neuroblastomas, paragangliomas, and renal carcinomas [22], and NPY2R agonists such as BIM-43004-1 suppress the growth of human pancreatic cancer xenografts in mice [23]. NPY4R is expressed in peripheral organs including the gastrointestinal tract, liver, pancreas, and heart [24]. The specific NPY4R agonist, BA-129 inhibits the proliferation of pancreatic cancer cells *in vitro* [25], and genetic and structural variations in NPY4R have been implicated in the pathogenesis of obesity [26].

Type 1 and 2 galanin receptors, type 1 tachykinin receptors, and type 1 somatostatin receptors, which are also neuropeptide GPCRs, are encoded by the *GALR1*, *GALR2*, *TACR1*, and *SSTR1* genes, respectively, and the methylation frequencies of these genes are 51.0%, 37.6%, 34.0%, and 64.0%, respectively [27–29]. In oral cancers, *GALR1* promoter hypermethylation significantly and inversely correlates with DFS time [29]. In oropharyngeal cancers, the odds ratio for recurrence is higher when the *GALR2* promoter is methylated versus unmethylated [29].

On the other hand, there is no association between *TACR1* or *SSTR1* methylation and prognosis in HNSCC patients regardless of tumor origin [27, 28].

Our study associates *NPY1R*, *NPY2R,* and *NPY4R* methylation with tumor recurrence in oral and oropharyngeal cancers. This finding may facilitate HNSCC screening and the development of surveillance algorithms. Simultaneous analysis of the methylation status of multiple neuropeptide GPCR-encoding genes will allow us to better predict tumor-related events, assess biological behavior, and design targeted therapies for HNSCCs.

GPCRs and their signal transduction networks affect physiological, immunological, and endocrinological processes and stem cell biology [4]. This is the first study

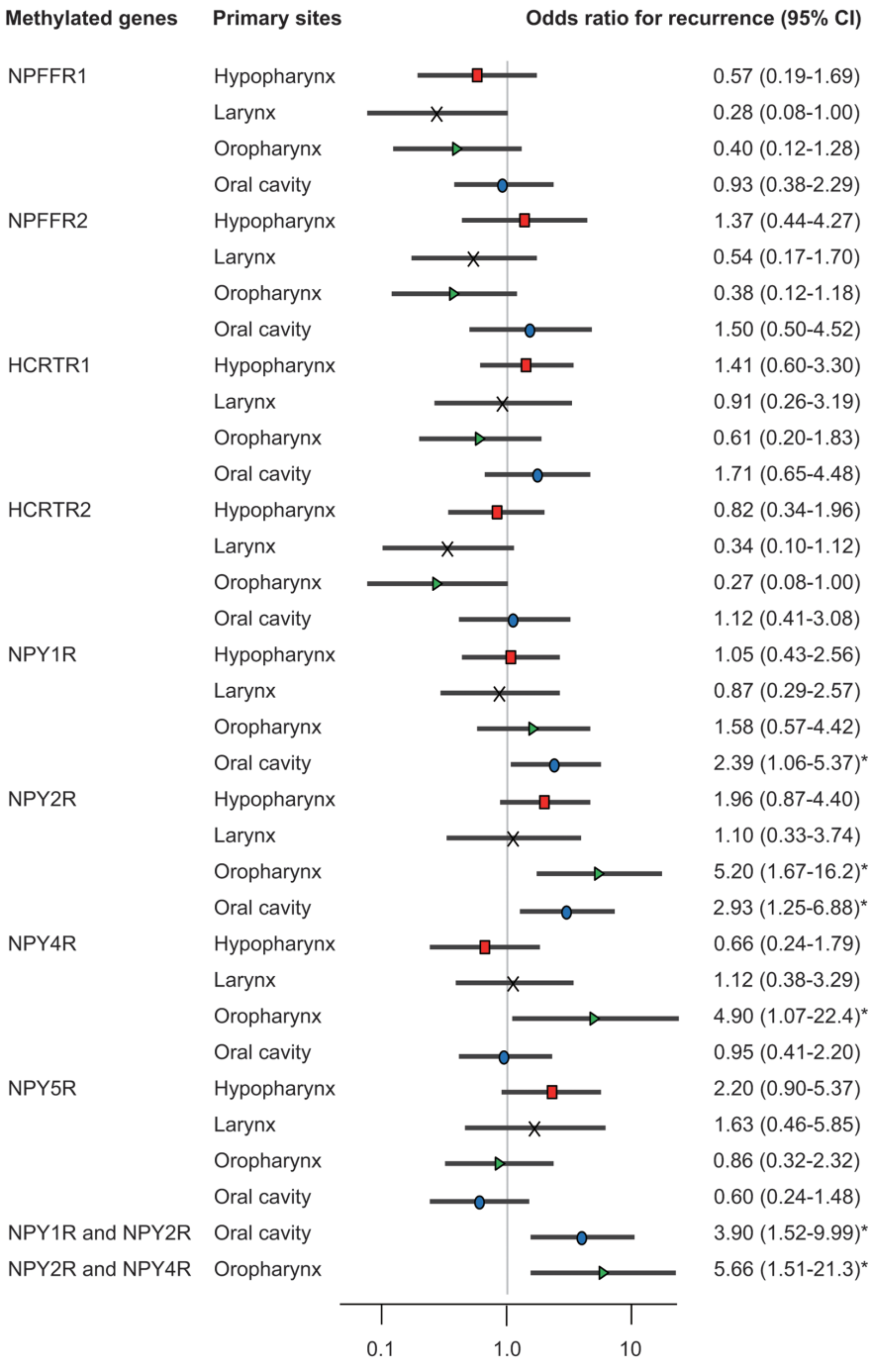

Figure 3: Risk of recurrence based on gene methylation in tumors with different origins. Odds ratios for recurrence were determined by using a Cox proportional hazards model adjusted for age (≥70 versus <70 years), sex, smoking status, alcohol intake, and stage (I–III versus IV). CI: confidence interval.

to show epigenetic regulation of eight neuropeptide receptor genes in HNSCC. It addresses the significant challenges unique to the identification of GPCR biomarkers and, as a GPCR-targeted study, may facilitate the identification of drugs for cancer prevention and treatment [30]. Both GPCRs and receptor-tyrosine kinases (RTKs) regulate extensive signaling networks, control multiple cell functions, and participate in many diseases including cancer [31]. Transactivation of epidermal growth factors, which are RTKs, by GPCRs has been reported; hence, specific disruption of the crosstalk between these receptor types, even without inhibition of their activities, should substantially impede disease progression [32].

Studies involving human specimens and high-throughput profiling platforms may be susceptible to measurement bias from a variety of sources. The present study suggests that the methylation status of the *NPY1R*, *NPY2R*, and *NPY4R* genes is an independent indicator of DFS in patients with oral and/or oropharyngeal cancers. Our findings support the use of methylation markers in patient selection for adjuvant therapy after initial surgical treatment and may aid oropharyngeal cancer screening and surveillance programs. However, they are preliminary and hence need to be validated in larger and more homogeneous HNSCC patient cohorts.

MATERIALS AND METHODS

Tumor samples

Two hundred and thirty-one 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. Pertinent information including age, sex, smoking status, alcohol consumption, lymph node status, tumor site, tumor size, and clinical stage was obtained from the patients' medical records (Supplementary Table 2). The male:female ratio in the patient cohort was 195:36. The mean age was 65.4 years (range, 32–93). Primary tumors were located in the hypopharynx (n = 59), larynx (n = 45), oropharynx (n = 58), or oral cavity (n = 69).

Quantitative methylation-specific PCR analysis

Extraction and bisulfite conversion of genomic DNA from 231 primary HNSCC and 36 noncancerous mucosal samples were performed using a MethylEasy Xceed Rapid DNA Bisulfite Modification Kit (TaKaRa, Tokyo, Japan) [33]. The methylation levels of the CpG islands in the promoters of the target genes were determined via quantitative methylation-specific PCR using the TaKaRa Thermal Cycler Dice TM Real Time System TP800 (TaKaRa). The primer sequences are listed in Supplementary Table 3. A standard curve was constructed by plotting known concentrations of serially diluted EpiScope™ Methylated HeLa gDNA (TaKaRa). 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 tumor sample and universal methylated DNA control, respectively, and ACTB-S and ACTB-FM represent *ACTB* (which encodes β-actin) methylation levels in the sample and control, respectively. Analysis was performed using the software (version 1.03A) for the Thermal Cycler Dice Real Time System TP800 according to the manufacturer's directions [34].

RNA extraction and quantitative reverse transcription (qRT-)PCR

Total RNA was isolated with RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized with ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Primer sequences are shown in Supplementary Table 4. Target mRNA expression was compared between samples by normalization to *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* mRNA expression.

Collection of publicly available data from TCGA

Aberrant DNA methylation data contained in TCGA (available in May 2017) were collected from the MethHC (http://methhc.mbc.nctu.edu.tw/php/index.php) [35] and cBioPortal (http://www.cbioportal.org/) databases [36] using the Infinium HumanMethylation450 platform (Illumina, Inc., San Diego, CA, USA) and are expressed as β values. The β value is a number between 0 (not methylated) and 1 (completely methylated) that represents the ratio of methylated allele intensity and overall intensity.

Statistical analysis

Receiver-operator characteristic (ROC) curve analysis was performed using the NMVs for 36 HNSCC and 36 adjacent normal mucosal samples and the Stata/SE 13.0 system (Stata Corporation, TX, USA). The area under the ROC curve indicated the optimal sensitivity and specificity cutoff levels for distinguishing between the methylation levels in normal and HNSCC tissue, and the NMV thresholds were calculated for each target gene. The cutoff values were used to determine the methylation frequencies of the target genes (Supplementary Figure 1). The overall methylation rates in the individual samples were determined by calculating the MIs [37, 38].

Associations between the variables were assessed by using Student's t-test. Disease-free survival (DFS) 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 survival probabilities, and the log-rank test was used to compare survival rates. The prognostic value of the methylation status was assessed by performing a multivariate Cox proportional hazards analysis adjusted for age (≥70 versus <70 years), sex, alcohol intake, smoking status, and tumor stage (I–III versus IV) [39]. Differences with $P < 0.05$ were considered significant. All statistical analyses were performed by using StatMate IV software (ATMS Co., Ltd., Tokyo, Japan).

Author contributions

Conceptualization: KM. Methodology: KM. Software: YM and TK. Validation: AI, SE, SH, RI, and MM. Formal analysis: DM. Investigation: TK. Resources: RI and MM. Resources: HM and MM. Writing – original draft: KM. Writing – review and editing: TK. Visualization: KM. Supervision: HM. Project administration: KM. Funding acquisition: KM. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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