Prostanoid receptor genes confer poor prognosis in head and neck squamous cell carcinoma via epigenetic inactivation

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Abstract

Background: Chronic inflammation is a risk factor for head and neck squamous cell carcinoma (HNSCC) and other diseases. Prostanoid receptors are clearly involved in the development of many types of cancer. However, their role is not simple and is poorly understood in HNSCC.

Methods: Methylation profiles of prostanoid receptor family genes were generated for tumour samples obtained from 274 patients with HNSCC, including 69 hypopharynx, 51 larynx, 79 oral cavity, and 75 oropharynx tumour samples, by quantitative methylation-specific PCR. Promoter methylation was then evaluated with respect to various clinical characteristics and patient survival.

Results: The mean number of methylated genes per sample was 2.05 ± 2.59 (range 0 to 9). Promoters of PTGDR1, PTGDR2, PTGER1, PTGER2, PTGER3, PTGER4, PTGFR, PTGIR, and TBXA2R were methylated in 43.8%, 18.2%, 25.5%, 17.5%, 41.2%, 8.0%, 19.3%, 20.4%, and 11.3% of the samples, respectively. Methylation indices for prostanoid receptor family genes tended to be higher as the number of TET methylation events increased. Patients with 5–9 methylated genes had a significantly lower survival rate than that of patients with 0–4 methylated genes (log-rank test, P = 0.007). In multivariate analyses, PTGDR1 methylation was most highly correlated with recurrence in patients with hypopharyngeal cancer (P = 0.014). A similar correlation was observed for PTGER4 in patients with laryngeal cancer (P = 0.046). Methylation of the PTGIR and TBXA2R promoters was positively correlated with recurrence in oropharyngeal cancer (P = 0.028 and P = 0.006, respectively). Moreover, Patients with 5–9 methylated genes were extremely lower of 5hmC levels (P = 0.035) and was correlated with increasing expression of DNMT3A and DNMT3B (P < 0.05 and P < 0.05, respectively).

Conclusion: We characterised the relationship between the methylation status of prostanoid receptor genes and recurrence in HNSCC. These results provide new perspectives for the development of molecular targeted treatment approaches.

Keywords: Prostanoid receptor genes, GPCRs, TET, Epigenetic markers, Q-MSP

Background

Clinical and epidemiological evidence suggests that chronic inflammation is a major risk factor for head and neck malignancies [1]. For example, patients with persistent human papilloma virus (HPV) infection, Epstein–Barr virus infection, or chronic inflammation (as observed in individuals with a cigarette smoking habit) face an increased lifetime risk for oropharyngeal cancer, nasopharyngeal cancer, or laryngeal cancer, respectively [2, 3]. Many studies have focused on cytokines and chemokines as mediators connecting chronic...
inflammation to head and neck cancer, but little is known about the involvement of prostanoid receptors [4]. To improve the survival rate for head and neck cancer, precision medicine approaches, improvements in diagnosis and prognosis, as well as the identification of novel targets and treatment strategies with minimal side effects are required.

G protein-coupled receptors (GPCRs) are the largest class of cell-surface receptors and are involved in many cancers, including head and neck squamous cell carcinoma (HNSCC) [5, 6]. GPCRs are modulated by a variety of endogenous and synthetic ligands and are major drug targets [7]. Key therapeutic applications involving GPCRs include opioid analgesics, antihistamines, anticholinergics, typical and atypical antipsychotics, antimigraine drugs, β2-agonists for asthma, and anti-hypertensives [8]. However, anti-cancer drugs that specifically target GPCRs are not currently available [9]. The prostanoid receptors represent the most notable family of validated pharmacological targets in a variety of diseases, including cancer [10].

Prostanoids derived from arachidonic acid through the cyclooxygenase (COX) pathway are particularly relevant. Prostaglandin H2 (PGH2) is the common cyclic-peroxide intermediate in the biosynthesis of prostanoids derived from arachidonic acid [11]. Prostanoids are a group of lipid mediators that include prostaglandins (PG) and thromboxanes (TX) [12]. Fatty acid COX converts arachidonic acid to PGH2, from which further prostanoids, PGD2, PGE2, PGF2α, PGI2 (prostacyclin), and thromboxane A2 (TXA2), may be enzymatically derived [13]. All nine prostanoid receptor genes [prostaglandin D2 receptors (PTGDR1 and PTGDR2), four prostaglandin E2 receptors (PTGER1, PTGER2, PTGER3 and PTGER4), the prostaglandin F receptor (PTGFR), the prostaglandin I2 receptor (PTGIR), and thromboxane A2 receptor (TBX2R)] encode neuropeptide receptors and belong to the GPCR Class Aα subgroup [14]. These nine prostanoid receptor genes have been implicated in the development of multiple types of cancer, but studies of the methylation status of all nine genes and their roles in the prognosis of HNSCC are lacking.

In this study, we provide that associations between the methylation status of nine prostanoid receptor genes and clinicopathological characteristics (e.g., tumour location and recurrence events) were also assessed. To our knowledge, this study is the first to link prostanoid receptor gene methylation to the genesis of HNSCC.

**Methods**

**Tumour samples**

Surgical HNSCC tumour and matched adjacent non-tumour tissues were obtained from 274 patients who underwent surgical resection at the Department of Otolaryngology/Head and Neck Surgery, Hamamatsu University School of Medicine (Hamamatsu, Shizuoka, Japan). Written informed consent was obtained from individual patients before surgery and the experimental protocol was approved by the Hamamatsu University School of Medicine (date of board approval: October 2, 2015, ethics code: 25-149). The ratio of males to females was 227:47. The mean age was 65.2 years (range, 32 to 90 years). Primary head and neck tumours included 69 hypopharyngeal carcinomas, 51 laryngeal carcinomas, 79 oropharyngeal carcinomas, and 75 oral cavity carcinomas (Additional file 1: Table S1).

**DNA extraction and modification**

DNA extraction from fresh tissue was performed using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Sodium bisulphite conversion was performed using the MethylEasy Xceed Rapid DNA Bisulfite Modification Kit (TaKaRa, Tokyo, Japan) following the manufacturer’s protocol.

**Quantitative methylation-specific PCR analysis (Q-MSP)**

Aberrant DNA methylation, which often occurs around the transcription start site (TSS) within a CpG island, was evaluated by Q-MSP. The sequences of primers used in this study are shown in Additional file 2: Table S2. Exon one or two and CpG sites within view of the promoter region relative to the TSS are presented in Additional file 3: Figure S1. A standard curve for Q-MSP was constructed by plotting five serially diluted standard solutions of EpiScope Methylated HeLa gDNA (TaKaRa). The normalized methylation value (NMV) was defined as follows: NMV = (prostanoid receptor gene-S/prostanoid receptor gene-FM)/(ACTB-S/ACTB-FM), where prostanoid receptor gene-S and prostanoid receptor gene-FM represent target gene methylation levels in the tumour sample and in the universal methylated DNA control, respectively. ACTB-S and ACTB-FM correspond to β-actin (ACTB) in the sample and the universally methylated DNA, respectively [15].

**Detection of high-risk HPV DNA by PCR**

To identify the HPV types, samples were also subjected to PCR using specific primers for HPV types 16, 18, 31, 33, 35, 52, and 58. The prevalence of HPV DNA was examined using the PCR HPV Typing Set (TaKaRa).

**ELISA for 5-hmC quantification**

The 5hmC content of genomic DNA was determined with a Quest 5-hmC DNA ELISA Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer’s instructions. The amount of 5-hmC was calculated as a...
The probability of survival can be evaluated by generating the Kaplan–Meier method and the log-rank test. Disease-free survival (DFS) was investigated to evaluate the associations between clinical variables and MI. The MI was defined as the number of genes with the greatest accuracy were determined based on sensitivity/specificity, as indicated in Additional file 4: Table S3. Methylation levels of all prostanoid receptor genes in primary HNSCCs were significantly higher than those in matched paired normal mucosal tissues, except for PTGER3 (Additional file 6: Fig. S3).

Results
Characterisation of 36 matched paired head and neck tumour samples and adjacent noncancerous mucosal samples
Promoter hypermethylation of nine prostanoid receptor genes exhibited distinct ROC curve profiles, which clearly differentiate cancer tissues from normal tissues (Additional file 5: Fig. S2). A specimen was classified as methylated when its NVM exceeded 0.161, 0.123, 0.048, 0.161, 0.502, 0.419, 0.368, 0.109, and 0.082 for PTGDR1, PTGDR2, PTGER1, PTGER2, PTGER3, PTGER4, PTGFR, PTGIR, and TBXA2R, respectively (Additional file 4: Table S3). Methylation levels of all prostanoid receptor genes in primary HNSCCs were significantly higher than those in matched paired normal mucosal tissues, except for PTGER3 (Additional file 6: Fig. S3).

Analysis of methylation status in HNSCC tissue samples
A Q-MSP analysis of the methylation status of nine prostanoid receptor genes was performed using 274 primary HNSCC samples. The methylation frequencies were as follows: PTGDR1 (43.8%), PTGDR2 (18.2%), PTGER1 (25.5%), PTGER2 (17.5%), PTGER3 (41.2%), PTGER4 (8.0%), PTGIR (19.3%), and TBXA2R (11.3%) (Fig. 1a). The average MI per sample was 2.05±2.59 (range 0 to 9) (Fig. 1b). No significant differences in MI were observed with respect to the age at disease onset, sex, alcohol consumption, smoking status, tumour size, lymph node status, clinical stage, or HPV status (Fig. 1c). We analysed the relationships of the methylation status of each prostanoid receptor gene with the clinical features of patients with HNSCC. PTGFR methylation was significantly correlated with age at onset (P = 0.043). Methylation levels of PTGDR2, PTGER1, and PTGIR promoters were associated with alcohol exposure (P = 0.042, P = 0.04, and P = 0.049, respectively). There was an association between methylation of the PTGDR1 and PTGER3 promoters and HPV status (P = 0.004 and P = 0.005, respectively). We found that the promoter methylation of all prostanoid receptor genes with the exception of TBXA2R was associated with recurrence events (Table 1).

Comparison of methylation frequencies between nine prostanoid receptor genes and ten-eleven translocation (TET) family genes
Mean differences in the MI of nine prostanoid receptor genes based on TET gene methylation events are illustrated in Fig. 2a. The MI was significantly higher in patients with methylation events at all TET genes (4.84±2.73), two TET gene methylation events (2.92±3.09), and one TET gene methylation event (1.94±2.03) than in patients with no TET gene methylation events (0.39±1.05; P < 0.01 for all comparisons) (Fig. 2b).
Survival analysis
The results of a Kaplan–Meier analysis of DFS are shown in Fig. 3. DFS did not differ between patients with methylated and unmethylated genes (Fig. 3d–h), with several notable exceptions, i.e., DFS was significantly shorter when the PTGDR1 (log-rank test, \( P = 0.019 \)), PTGDR2 (log-rank test, \( P = 0.025 \)), PTGER1 (log-rank test, \( P = 0.024 \)), and TBXA2R (log-rank test, \( P = 0.041 \)) promoters were methylated (Fig. 3a–c, i). DFS in patients with 5–9 methylated genes was lower than that in the group with 0–4 methylated genes (35.4% versus 59.0%; log-rank test, \( P = 0.007 \); Fig. 3j, Additional file 7: Table S4).

Site-specific analysis of the methylation status
Site-specific methylation frequencies across nine genes for the hypopharynx, larynx, oropharynx, and oral cavity are shown in Fig. 4a. MI levels were significantly higher in patients with hypopharyngeal cancer than in patients with oral cavity cancer (\( P = 0.020 \)) (Fig. 4b). Among 69 cases with hypopharyngeal cancer, the DFS rate in those with PTGDR1 methylation was similar to that in the unmethylated group (log-rank test, \( P = 0.011 \); Additional file 8: Fig. S4A). Patients with laryngeal cancer and methylated PTGER4 promoters had a relatively short DFS (log-rank test, \( P = 0.020 \); Additional file 8: Fig. S4B). Additional analysis including only patients with oropharyngeal cancer (n = 79) revealed a shorter DFS for methylated than for unmethylated PTGIR and TBXA2R (log-rank test, \( P = 0.003 \) and \( P = 0.009 \), respectively; Additional file 8: Fig. S4C, D).

Stratification analysis
The relation between the methylation status and risk of recurrence was analysed by a multivariate analysis using a Cox proportional hazards regression model adjusted for age, sex, smoking status, alcohol consumption, and clinical stage. For 274 patients with PTGDR1 promoter methylation, the adjusted odds ratio (OR) for recurrence was 1.58 [95% confidence interval (CI) 1.06–2.33, \( P = 0.023 \)]. In patients with hypopharyngeal cancer, PTGDR1 promoter methylation was significantly associated with recurrence (OR = 2.76, 95% CI 1.23–6.18, \( P = 0.014 \)). For patients with laryngeal cancer with a methylated PTGER4 promoter, the OR was 5.04 (95% CI 1.03–24.70; \( P = 0.046 \)). Methylation statuses of the PTGIR and TBXA2R promoters were positively correlated with recurrence in patients with oropharyngeal cancer (OR, 2.99; 95% CI 1.13–7.92; \( P = 0.028 \) and OR, 5.21; 95% CI 1.63–16.67; \( P = 0.006 \), respectively) (Fig. 5).

Comparison of methylation frequencies between nine prostanoid receptor genes and other epigenetic factors
The 5-hmC level showed the greatest decrease when prostanoid receptor genes were 9 to 5 methylation events
Table 1 Distribution of methylation status by selected epidemiologic and clinical characteristics

| Gene    | Methylation status | Characteristics | Age            | Gender | Smoking status | Alcohol exposure |
|---------|--------------------|----------------|----------------|--------|----------------|------------------|
|         |                    |                | Overall (%)    | < 65   | > 65 | P          | Female | Male | P          | Smoker | Non smoker | Drinker | Non drinker | P          |
| PTGDR1  | Yes                | 120 (43.8)     | 50             | 70     | 16  | 104         | 97     | 23   | 105       | 15     | 104         | 97       | 23          | 105       |
|         | No                 | 154 (56.2)     | 63             | 91     | 1   | 123         | 111    | 43   | 111       | 42     | 112         | 112      | 42          | 112       |
| PTGDR2  | Yes                | 50 (18.2)      | 16             | 34     | 7   | 43          | 42     | 8    | 42         | 8      | 44          | 44       | 6           |
|         | No                 | 224 (81.8)     | 97             | 127    | 0.156 | 40 | 184 | 0.678 | 166 | 58 | 0.199 | 165 | 59 | 0.042* |
| PTGER1  | Yes                | 70 (25.5)      | 30             | 40     | 8   | 62          | 55     | 15   | 55         | 15     | 60          | 60       | 10          |
|         | No                 | 204 (74.5)     | 83             | 121    | 1   | 165         | 153    | 51   | 153        | 55     | 153         | 153      | 51          | 153       |
| PTGER2  | Yes                | 48 (17.5)      | 18             | 30     | 10  | 38          | 37     | 11   | 37         | 11     | 37          | 37       | 11          |
|         | No                 | 226 (82.5)     | 95             | 131    | 0.630 | 37 | 189 | 1     | 171 | 55 | 1     | 170       | 56 | 0.457     |
| PTGER3  | Yes                | 113 (41.2)     | 49             | 64     | 17  | 96          | 87     | 26   | 87         | 26     | 86          | 86       | 27          |
|         | No                 | 161 (58.8)     | 64             | 97     | 1   | 131         | 121    | 40   | 121        | 40     | 123         | 123      | 38          | 1         |
| PTGER4  | Yes                | 22 (8.0)       | 6              | 16     | 4   | 18          | 18     | 4    | 18         | 4      | 18          | 18       | 4           |
|         | No                 | 252 (92.0)     | 107            | 145    | 0.183 | 43 | 209 | 1     | 190 | 62 | 0.610 | 191       | 61 | 0.612     |
| PTGFR   | Yes                | 53 (19.3)      | 15             | 38     | 8   | 45          | 44     | 9    | 44         | 9      | 46          | 46       | 7           |
|         | No                 | 221 (80.7)     | 98             | 123    | 0.043* | 39 | 182 | 0.839 | 164 | 57 | 0.212 | 163       | 58 | 0.049*    |
| PTGIR   | Yes                | 56 (20.4)      | 20             | 36     | 8   | 48          | 46     | 10   | 46         | 10     | 48          | 48       | 8           |
|         | No                 | 218 (79.6)     | 93             | 125    | 0.366 | 39 | 179 | 0.691 | 162 | 56 | 0.293 | 161       | 57 | 0.078     |
| TBX2AR  | Yes                | 31 (11.3)      | 10             | 21     | 4   | 27          | 27     | 4    | 27         | 4      | 27          | 27       | 4           |
|         | No                 | 243 (88.7)     | 103            | 140    | 0.355 | 43 | 200 | 0.620 | 181 | 62 | 0.179 | 182       | 61 | 0.346     |

| Gene    | Tumor size | Lympho-node status | Stage | HPV status | Recurrence events |
|---------|------------|---------------------|-------|------------|-------------------|
|         | T1–2       | T3–4 | P        | N0 | N+ | P        | I, II | III, IV | P        | Negative | Positive | P        | Negative | Positive | P        |
| PTGDR1  | 54         | 66 | 0.274 | 47 | 73 | 0.24 | 24 | 36 | 0.557 | 105 | 36 | 0.004* | 127 | 32 | < 0.001* |
|         | 80         | 74 | 129 | 61 | 93 | 1 | 96 | 118 | 0.557 | 95 | 36 | 0.004* | 127 | 32 | < 0.001* |
| PTGDR2  | 23         | 27 | 0.755 | 92 | 132 | 0.265 | 41 | 173 | 0.572 | 156 | 45 | 0.018 | 152 | 73 | 0.005* |
|         | 111        | 113 | 0.269 | 81 | 123 | 0.888 | 55 | 159 | 1 | 141 | 40 | 0.297 | 145 | 60 | < 0.001* |
| PTGER1  | 30         | 40 | 0.525 | 89 | 137 | 1 | 38 | 176 | 1 | 158 | 45 | 0.164 | 155 | 72 | < 0.001* |
|         | 104        | 100 | 0.462 | 65 | 96 | 0.708 | 90 | 124 | 0.658 | 101 | 37 | 0.005* | 136 | 28 | < 0.001* |
| PTGER2  | 21         | 27 | 52 | 61 | 70 | 23 | 23 | 37 | 99 | 14 | 38 | 72 | 9 | 13 |
|         | 113        | 113 | 82 | 79 | 0.462 | 65 | 96 | 0.708 | 90 | 124 | 0.658 | 101 | 37 | 0.005* | 136 | 28 | < 0.001* |
| PTGER3  | 52         | 61 | 43 | 70 | 23 | 23 | 37 | 99 | 14 | 38 | 72 | 9 | 13 |
|         | 123        | 129 | 1 | 96 | 156 | 0.172 | 16 | 198 | 1 | 180 | 49 | 0.266 | 165 | 87 | 0.035* |
| Gene   | Tumor size | Lympho-node status | Stage | HPV status | Recurrence events |
|--------|------------|--------------------|-------|------------|-------------------|
|        | T1–2  | T3–4  | P† | N0 N+ | P† | I, II | III, IV | P† | Negative | Positive | P‡ | Negative | Positive | P‡ |
| PTGFR  | 26    | 27    |    | 19    | 34    | 12    | 48    |    | 46      | 7       |    | 22      | 31       |    |
|        | 108   | 113   | 1  | 89    | 132   | 41    | 173   | 1  | 154     | 44      | 0.180 | 152     | 69       | < 0.001* |
| PTGIR  | 28    | 28    |    | 16    | 40    | 9     | 51    |    | 48      | 8       |    | 24      | 32       |    |
|        | 106   | 112   | 1  | 92    | 126   | 47    | 167   | 0.280 | 152     | 43      | 0.259 | 150     | 68       | < 0.001* |
| TBX2A2R| 10    | 21    |    | 13    | 18    | 7     | 53    |    | 27      | 4       |    | 16      | 15       |    |
|        | 124   | 119   | 0.057 | 95    | 148   | 1    | 24    | 190   | 1    | 173     | 47      | 0.346 | 158     | 85       | 1    |

* P < 0.05, †Chi squared test
The methylation status of prostanoid receptor gene promoters was estimated in an additional 516 HNSCC samples and 50 normal samples from TCGA. The average β-values (indicating promoter methylation) for the nine genes were significantly higher in the HNSCC samples than in the normal samples (P < 0.05), except for PTGER4 and TBXA2R (Additional file 10: Fig. S6). The expression of prostanoid receptor genes were significantly higher in IL-6 mRNA high expression group (P < 0.05), except for PTGER1 gene. IL-11 expression was positively correlated with PTGDR1 and TBXA2R expression (P = 0.001 and P < 0.001, respectively). Expression of RANKL was concurrently associated with all prostanoid receptor genes expression (Additional file 11: Table S5).

**Discussion**

Epigenetic modifications of prostanoid receptor genes may contribute to tumour development and recurrence. We analysed the methylation statuses of genes encoding neuropeptide GPCRs in 274 HNSCCs originating in the hypopharynx, larynx, oropharynx, or oral cavity. We also compared the methylation status of genes in matched HNSCC and normal samples using data from TCGA. We found that the aberrant methylation of some prostanoid receptor gene promoters is positively correlated with recurrence in patients with HNSCC. In addition, a site-specific analysis revealed that abnormal CpG island hypermethylation was independently associated with aggressive clinical behaviour.

Cancer may be related to chronic inflammation associated with persistent infections, immune-mediated damage, or prolonged exposure to irritants. Genetic and epigenetic alterations underlying carcinogenesis inevitably modify tissue homeostasis and may induce a chronic inflammatory response. Over 20 years ago, non-steroidal anti-inflammatory drugs (NSAIDs) were reported to have anti-colon cancer effects [18]. NSAIDs, which are potent inhibitors of COX, exert chemopreventive effects in cancer development [19]. Numerous epidemiological studies have shown that the regular intake of the NSAID aspirin, an inhibitor of COXs, substantially reduces both the incidence and progression of several prevalent cancers [20]. Abundant epidemiological and preclinical/clinical studies have demonstrated that celecoxib, a specific COX-2 inhibitor, is related to the suppression of cancer cell proliferation and a decrease in cancer incidence [21]. COX-1 is constitutively expressed in many tissues and regulates basal levels of prostaglandins [22]. COX-2 is responsible for the release of prostaglandins after an infection, injury, or in cancer development [23]. In HNSCC, IL-1 released by tumour cells plays a key role in inducing
Fig. 3 Kaplan–Meier survival curves based on 274 patients with HNSCC according to the methylation status of the nine prostanoid receptor genes. DFS with respect to a PTGDR1, b PTGDR2, c PTGER1, d PTGER2, e PTGER3, f PTGER4, g PTGFR, h PTGIR, and i TBXA2R in the case of methylated (red lines) and unmethylated (blue lines) genes. j Combined analysis of the nine genes. Blue line: patients with 0–4 methylated genes; red line: patients with 5–9 methylated genes. *P < 0.05
Fig. 4  Site-specific methylation frequencies for nine prostanoid receptor genes. a Comparison of methylation statuses of the promoters of the nine prostanoid receptor genes in patients with hypopharyngeal, laryngeal, oropharyngeal, or oral cancer. Filled boxes indicate the presence of methylation, and open boxes indicate the absence of methylation. b The mean MI values for various groups were compared using Student’s t-tests. *P < 0.05
| Methylated genes | Primary sites | Odds ratio for recurrence (95%CI) |
|------------------|--------------|----------------------------------|
| PTGDR1           | Full panel   | 1.58 (1.06-2.33)*                |
|                  | Hypopharynx  | 2.76 (1.23-6.18)*                |
|                  | Larynx       | 1.47 (0.61-3.54)                 |
|                  | Oropharynx   | 1.78 (0.70-4.55)                 |
|                  | Oral cavity  | 0.83 (0.27-1.46)                 |
| PTGDR2           | Full panel   | 1.41 (0.88-2.27)                 |
|                  | Hypopharynx  | 0.85 (0.26-1.66)                 |
|                  | Larynx       | 1.02 (0.33-3.18)                 |
|                  | Oropharynx   | 2.73 (0.99-7.55)                 |
|                  | Oral cavity  | 1.91 (0.74-4.90)                 |
| PTGER1           | Full panel   | 1.35 (0.87-2.07)                 |
|                  | Hypopharynx  | 0.80 (0.35-1.83)                 |
|                  | Larynx       | 1.15 (0.44-3.07)                 |
|                  | Oropharynx   | 2.26 (0.90-5.71)                 |
|                  | Oral cavity  | 1.91 (0.75-4.81)                 |
| PTGER2           | Full panel   | 1.20 (0.73-1.97)                 |
|                  | Hypopharynx  | 1.26 (0.50-3.15)                 |
|                  | Larynx       | 0.81 (0.28-2.39)                 |
|                  | Oropharynx   | 1.26 (0.33-4.81)                 |
|                  | Oral cavity  | 1.01 (0.36-2.82)                 |
| PTGER3           | Full panel   | 1.05 (0.70-1.55)                 |
|                  | Hypopharynx  | 1.12 (0.52-2.41)                 |
|                  | Larynx       | 1.18 (0.43-3.26)                 |
|                  | Oropharynx   | 0.88 (0.33-2.38)                 |
|                  | Oral cavity  | 0.63 (0.28-1.37)                 |
| PTGER4           | Full panel   | 1.32 (0.66-2.62)                 |
|                  | Hypopharynx  | 0.22 (0.03-1.66)                 |
|                  | Larynx       | 5.04 (1.03-24.7)*                |
|                  | Oropharynx   | 1.06 (0.21-5.29)                 |
|                  | Oral cavity  | 2.70 (0.90-8.10)                 |
| PTGFR            | Full panel   | 1.26 (0.78-2.03)                 |
|                  | Hypopharynx  | 0.67 (0.26-1.70)                 |
|                  | Larynx       | 0.89 (0.29-2.76)                 |
|                  | Oropharynx   | 2.17 (0.78-6.05)                 |
|                  | Oral cavity  | 1.79 (0.66-4.80)                 |
| PTGIR            | Full panel   | 1.22 (0.76-1.94)                 |
|                  | Hypopharynx  | 0.74 (0.29-1.91)                 |
|                  | Larynx       | 0.97 (0.34-2.80)                 |
|                  | Oropharynx   | 2.99 (1.13-7.92)*                |
|                  | Oral cavity  | 0.79 (0.28-2.21)                 |
| TBX2A2R          | Full panel   | 1.63 (0.95-2.79)                 |
|                  | Hypopharynx  | 1.32 (0.50-3.52)                 |
|                  | Larynx       | 1.07 (0.24-4.75)                 |
|                  | Oropharynx   | 5.21 (1.63-16.7)*                |
|                  | Oral cavity  | 1.33 (0.44-3.97)                 |

**Fig. 5** Risk of recurrence based on gene methylation in tumours of different origins. Odds ratios for recurrence were determined using a Cox proportional hazards model adjusted for age (≥65 vs. <65 years), sex, smoking status, alcohol intake, and tumour stage (I–II vs. III–IV). CI = confidence interval. *P < 0.05
the expression of COX-2 in fibroblasts [11]. Secretion of TGF-β and PGE2 by the HNSCC cells was increased following EGFR inhibition [24]. IL-6, TNF-α and PGE2 produced by primary oral keratinocytes and carcinoma cells may induce oral mucosal inflammation [25]. Recent studies continue to support the prostanoid pathway as a promising target for future HNSCC therapies.

Prostanoids, including PGD2, PGE2, PGF2α, PGI2, and TXA2, activate nine GPCRs, namely PTGER1, PTGDR1, TXA2R, PTGFR, PTGIR, and TBXA2R. PTGDRI downregulation by DNA hypermethylation is correlated with colorectal cancer development [26, 27]. PTGDRI methylation from cervical scraping is a promising marker of endometrial cancer and ovarian cancer [28]. PTGER1 shows a strong association with DNA methylation in non-functioning adenocortical adenoma [29]. The expression of PTGER2 is often silenced in neuroblastoma cell lines by epigenetic mechanisms [30]. Increased DNA methylation of PTGER2 is associated with the progression of neuroblastomas [30], non-small cell lung cancer [31], and cervical cancer tissue [32], suggesting that the aberrant methylation of this gene regulates cell proliferation. Cebola et al. detected PTGER3 and PTGFR hypermethylation in a high proportion of colorectal cancer cases, suggesting that DNA methylation is an important mechanism involved in the deregulation of this pathway [33]. The measurement of PTGER4 methylation in plasma DNA obtained by minimally invasive sampling can be used to detect malignant lung disease [34]. The loss of methylation and activation of PTGER4 can explain the acquisition of endocrine therapy resistance and is a therapeutic target for breast cancer [35].

Cancers of the upper aerodigestive tract account for the majority of squamous cell carcinomas, which develop in the epithelial linings of the oral cavity, pharynx, and larynx [36]. There are several subclassifications based on anatomic location, aetiology, and molecular findings [37]. Head and neck cancers arise from a multistep process involving the accumulation of genetic and epigenetic alterations [38]. DNA methylation is a frequent and key epigenetic mechanism underlying the regulation of processes associated with neoplastic transformation [38]. Recently, TET proteins have been identified as important epigenetic modifiers via their dioxygenase activity [39]. TET expression and activity are inhibited by genetic mutations and high methylation of their own promoters [40]. Our data showed that increased DNA methylation of TET genes is correlated with the accumulation of prostanoid receptor genes with aberrant methylation; this may be a meaningful DNA methylation event in HNSCC progression. Furthermore, the groups of high MI were extremely low of 5hmC levels and was correlated with increasing expression of DNMT3A and DNMT3B.

To our knowledge, our study is the first to suggest that PTGDRI, PTGER4, PTGIR, and TBXA2R methylation is associated with worse DFS and may be a critical event in hypopharyngeal cancers, laryngeal cancers, oropharyngeal cancers, and oral cancers, respectively. However, our results obtained from human specimens and high-throughput profiling platforms may be susceptible to measurement bias from various sources. The current study continues to support the prostanoid receptor as a promising target for future HNSCC therapies.

We systematically evaluated the methylation status of the promoters of nine prostanoid receptor genes and the relationship between methylation and clinical characteristics in HNSCC samples. We identified a novel prognostic biomarker based on promoter DNA methylation changes in operable HNSCC to identify patients at high risk of recurrence and provide complementary epigenetic characterization of this tumour type. Collectively, these data demonstrate the functional roles of the epigenetic regulation of prostanoid receptors and show that these loci are potential targets for epigenetic therapies for inflammatory disorders, such as HNSCC.

Conclusion

We determined the relationship between the methylation status of prostanoid receptor genes and recurrence in HNSCC, providing new perspectives for the development of molecular targeted therapeutic approaches. To our knowledge, our study provides the first evidence for an association between PTGDRI, PTGER4, PTGIR, and TBXA2R methylation and worse survival in hypopharyngeal cancers, laryngeal cancers, oropharyngeal cancers, and oral cancers, respectively. This study involving human specimens and high-throughput profiling platforms may be susceptible to measurement bias from various sources; accordingly the use of methylation markers in clinical practice requires further testing in prospective studies with larger HNSCC cohorts.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12967-020-02214-1.

Additional file 1: Table S1. Baseline characteristics of the HNSCC patients.
Additional file 2: Table S2. Q-MSM primer list.
Additional file 3: Fig. S1. Schematic representation of PTGDRI, PTGDRI, PTGER4, PTGIR, and TBXA2R genes. CPG sites are within the expanded views of the promoter region. Vertical lines, individual CPG sites; black box, relative location of the primers used for Q-MSM: bent arrow, translation start site (ATG).
Additional file 4: Table S3. Results of the ROC curve analysis, the sensitivity, specificity, and cutoff value.
Additional file 5: Fig. S2. Receiver operating characteristic (ROC) curves for the methylation markers in cancer tissue versus adjacent normal mucosal tissue. Based on the ROC curve analysis, Area Under Curves (AUCs) are 0.6767 for PTGER1 (A), 0.6265 for PTGER2 (B), 0.6574 for PTGER1 (C), 0.6154 for PTGER2 (D), 0.4784 for PTGER1 (E), 0.5405 for PTGER4 (F), 0.6289 for PTGFR (G), 0.6736 for PTGFR (H) and 0.6605 for TXA2R (I).

Additional file 6: Fig. S3. Hypermethylation patterns in 36 matched pairs of head and neck tumors and adjacent normal mucosal tissues. The NMsVs for the PTGDR1 (A), PTGDR2 (B), PTGIR1 (C), PTGIR2 (D), PTGIR3 (E), PTGER4 (F), PTGFR (G), PTGIR (H) and TXA2R (I) promoters were significantly higher in head and neck tumor tissues (T) than in paired adjacent normal mucosal tissue (N). The differences were significant as determined by the Student’s t-test. *P < 0.05.

Additional file 7: Table S4. Results of log-rank tests for effect of number of methylated genes on disease free survival in 274 HNSCC.

Additional file 8: Fig. S4. Distribution of expression levels in TCGA Additional file 11: Table S5. from TCGA database. *P < 0.05.

Additional file 9: Fig. S5. Comparison of methylation frequencies between nine prostanoid receptor genes and other epigenetic factors. (A) ShmC levels, (B) DNMT3A mRNA levels, (C) DNMT3B mRNA levels. *P < 0.05. The data are shown as the mean ± SE.

Additional file 10: Fig. S6. Methylation status of the five neuropeptide receptor genes in HNSCC and normal samples in TCGA database. The methylation data for PTGDR1, PTGDR2, PTGIR1, PTGIR2, PTGIR3, PTGER4, PTGFR, PTGIR and TXA2R in HNSCC and normal samples were collected from TCGA database. *P < 0.05.

Additional file 11: Table S5. Distribution of expression levels in TCGA cohort of HNSCC.

Ethics approval and consent to participate The research methodology employed in this study was approved by The Institutional Review Board of the Hamamatsu University School of Medicine. All study subjects provided written informed consent.

Consent for publication Consent for publication was obtained from all patients.

Competing interests The authors declare that they have no competing interests.

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