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KIF1A and EDNRB are differentially methylated in primary HNSCC and salivary rinses

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Abstract

Silencing of tumor suppressor genes plays a vital role in head and neck carcinogenesis. In this study, we aimed to evaluate to the utility of aberrant promoter hypermethylation for detection in a panel of 10 genes (*KIF1A, EDNRB, CDH4, TERT, CD44, NISCH, PAK3, VGF, MAL* and *FKBP4*) in head and neck squamous cell carcinoma (HNSCC) via a candidate gene approach. We investigated methylation of the gene promoters by bisulfite modification and quantitative methylation-specific PCR (Q-MSP) in a preliminary study of a limited cohort of salivary rinses from healthy subjects ($n = 61$) and patients with HNSCC ($n = 33$). The methylation status of 2 selected genes (*EDNRB* and *KIF1A*) were then analyzed in 15 normal mucosa samples from a healthy population, 101 HNSCC tumors and the corresponding salivary rinses from 71 out of the 101 HNSCC patients were collected before treatment. The promoter regions of *CDH4, TERT, VGF, MAL, FKBP4, NISCH* and *PAK3* were methylated in normal salivary rinses while no methylation of *CD44* was observed in either normal salivary rinses or tumor samples. However, *KIF1A* and *EDNRB* were methylated in 98 and 97% of primary HNSCC tissues respectively and were only methylated in 2 and 6.6% of normal salivary rinses. In addition, *KIF1A* and *EDNRB* were methylated in 38 and 67.6% of salivary rinses from HNSCC patients, respectively. Promoter hypermethylation of *KIF1A* and *EDNRB* is a frequent event in primary HNSCC, and these genes are preferentially methylated in salivary rinses from HNSCC patients. *KIF1A* and *EDNRB* are potential biomarkers for HNSCC detection.

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Additional Supporting Information may be found in the online version of this article.

Potential Conflicts of Interest: Under a licensing agreement between Oncomethylome Sciences, SA and The Johns Hopkins University, Dr. D. Sidransky is entitled to a share of royalty received by the University upon sales of diagnostic products described in this article. Dr. D. Sidransky owns Oncomethylome Sciences, SA stock, which is subject to certain restrictions under University policy. Dr. Sidransky is a paid consultant to Oncomethylome Sciences, SA and is a paid member of the company's Scientific Advisory Board. The Johns Hopkins University in accordance with its conflict of interest policies is managing the terms of this agreement. Dr. N. Liegeois is the President and Founder of Meridian Skin Care Ltd.

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Keywords

methylation; saliva; head and neck cancer; *KIF1A*; *EDNRB*

Among human malignancies, head and neck cancer is the sixth most common cancer in the world.[1] More than 40,000 new cases of head and neck squamous cell carcinoma (HNSCC) are diagnosed in the United States each year, with a mortality rate of 12,000 U.S. deaths annually. Survival rates have not improved significantly for patients with HNSCC in the past 30 years despite active clinical and basic science research addressing this issue. Molecular detection of HNSCC in body fluids has the potential to improve post-treatment surveillance, provide prognostic information, and influence therapy. Body fluids can potentially carry whole cells as well as protein, DNA and RNA species that allow for detection of cellular alterations related to cancer. In previous studies, body fluids such as sputum for lung cancer,[2] urine for urologic tumors,3 salivary rinses for HNSCC,4–7 and breast fluid for breast cancer[8] have been used in multiple detection strategies.[9–13]

Silencing of tumor suppressor genes by means of promoter hypermethylation plays a role in head and neck carcinogenesis.[5] Measuring promoter hypermethylation by using real time quantitative methylation-specific PCR (Q-MSP) allows an objective, robust, and rapid assessment of promoter methylation status. The ability to quantify methylation provides the potential for determination of a clinically meaningful threshold value of methylation to improve sensitivity and specificity in detection of tumor-specific signals.[14–17]

In this study, we evaluated the epigenetic changes in head and neck squamous cell carcinoma (HNSCC) by investigating promoter hypermethylation in a panel of 10 genes (*KIF1A, EDNRB, CDH4, TERT, CD44, NISCH, PAK3, VGF, MAL* and *FKBP4*) via a candidate gene approach. We sought to determine if a novel panel of promoter hypermethylation markers would result in an improved ability to detect epigenetic changes associated with HNSCC in salivary rinses from patients with HNSCC.

In this study, we were able to show differential promoter hypermethylation in HNSCC patients compared with normal individuals' salivary rinses. We then compared promoter hypermethylation of *KIF1A* with that of *EDNRB*, finding a significant association between methylation of promoter regions of these genes. Promoter hypermethylation for both of these genes was then examined for association with clinical parameters.

Material and Methods

Samples

After institutional review board approval, and obtaining appropriate informed consent, the patients and control population from healthy subjects enrolled in a community screening study were recruited from the Johns Hopkins School of Medicine, Department of Otolaryngology-Head and Neck Surgery. Hundred and one tumor and the corresponding pretreatment salivary rinse samples collected before any treatment for 71 of these tumors were obtained from HNSCC patients. For the control population, 61 salivary rinse samples and 15 normal mucosal tissue samples from a healthy cohort were collected. All subjects were administered a confidential written survey of risk factors for upper aerodigestive tract malignancies, including alcohol and tobacco. Smoking was defined as use of tobacco, chewable or smoked, for at least 1 year continuously. Alcohol use was defined as intake of more than 2 alcoholic drinks per day. In this study, salivary rinses were obtained by brushing oral cavity and oropharyngeal surfaces with an exfoliating brush followed by rinse and gargle with 20 mL normal saline solution. The brush was gently agitated to release the obtained material into saline. After centrifugation, the

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supernatant was discarded and DNA was isolated from the pellet. Tumors were snap frozen and microdissected on a cryostat to at least 75% purity. The possibility of contamination has been precluded by careful analysis and preselection of the malignant tissue regions by microdissection and further analysis by an experienced pathologist. DNA from 61 salivary rinse samples from noncancer individuals were analyzed as a control, to investigate the normal promoter methylation status of *KIF1A* (n = 47), *EDNRB* (n = 45), *CDH4* (n = 20), *TERT* (n = 46), *CD44* (n = 46), *NISCH* (n = 46), *PAK3* (n = 46), *VGF* (n = 21), *MAL* (n = 21) and *FKBP4* (n = 21) genes. The methylation status of 2 selected genes, *EDNRB* and *KIF1A*, were analyzed in 101 fresh tumors and 71 salivary rinse samples from patients with head and neck cancer and 15 normal mucosa samples from healthy individuals.

DNA extraction and bisulfite treatment

DNA was isolated as described previously.[7] In brief, DNA was obtained by phenol/chloroform extraction after overnight incubation with proteinase K at 37°C. DNA from tumor and control samples was subjected to bisulfite treatment using Epitect Bisulfite Modification kit (Qiagen, Valencia, CA) as per the manufacture's protocol.

Bisulfite sequencing

Bisulfite-treated DNA was amplified for the 5′ region that included at least a portion of the CpG island within 1–2 kb of the first exon of the genes, *CD44* and *CDH4*, using primer sets (Table S1). The primers for bisulfite sequencing were designed to hybridize to regions in the promoter without CpG dinucleotides. PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Each amplified DNA sample was sequenced by the Applied Biosystems 3700 DNA analyzer using nested, forward or reverse primers and BD terminator dye (Applied Biosystems, Foster City, CA).

Quantitative methylation specific PCR

Primers and probes were obtained from literature for *KIF1A, NISCH, PAK3*,[18] *CD44* and *EDNRB*,[19] *TERT*,[20] *MAL*, *FKBP4* and *VGF*[21] genes, and the internal control β-*actin* gene (*ACTB*),[22] and are given in Supplementary Table S2. Primer and probe sequences were determined by Methprimer program showing the CpG islands in the promoter regions of *CDH4* gene after bisulfite sequencing (Table S2). Lymphocytes obtained from a healthy individual were *in vitro* methylated using excess SssI methyltransferase (New England Biolabs Inc., Beverly, MA) to generate completely methylated DNA that was used as a positive control standard. To quantitate the relative percent of methylation, we computed the ratio between the QMSP values of the gene of interest relative to an internal control, *ACTB* (gene of interest/reference gene ×100). Fluorogenic PCR is carried out in a reaction volume of 20 μL consisting of 600 nM of each primer; 200 nM of probe; 0.6 U of platinum Taq polymerase (Invitrogen, Carlsbad, CA); 200 μM of each dATP, dCTP, dGTP, and dTTP; ×1 Rox Dye reference and ×1 Buffer [16.6 mM of ammonium sulfate; 67 mM of Trizma (Sigma, St Louis, MO); 6.7 mM of Magnesium chloride; 10 mM of mercaptoethanol; and 0.1% dimethylsulfoxide].

Bisulfite treated DNA of 30 ng was used in each real-time QMSP reaction. Amplifications are carried out in 384-well plates in a 7900 Sequence Detector System (Perkin-Elmer Applied Biosystems, Norwalk, CT) and are analyzed by SDS 2.3 (Sequence Detector System) (Applied Biosystems). Each reaction was performed in triplicate.

Data analysis

Statistical analyses were performed using S-PLUS® 8.0 for Windows Enterprise Developer. Methylation levels of *KIF1A* and *EDNRB* between healthy subjects and HNSCC patients were

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compared using Wilcoxon rank test. Spearman correlations between *KIF1A* and *EDNRB* methylation level were calculated and tested against zero. Binary indicators of *KIF1A* and *EDNRB* methylation in HNSCC patients' salivary rinses were used to study their association with cancer mortality. Such associations were not studied in tumor samples since all except 2 and 3 patients for *KIF1A* and *EDNRB* respectively, had methylation in both genes. For each prognostic factor, proportional hazards model was used to estimate the hazard ratio and the corresponding 95% confidence interval (CI). P-values were obtained from the likelihood ratio test for continuous covariates and log-rank test for binary covariates. A multivariate Cox proportional hazards model was used to investigate the joint association of several risk factors with cancer mortality. All tests were two-sided with significance level set at $p = 0.05$.

Results

Clinicopathologic characteristics of control subjects and patients with HNSCC

Table 1 describes the sample populations used in this study. The ages of patients from which the normal mucosal samples were obtained are slightly lower than the population of head and neck cancer patients, mean ages 43 years (range, 22–65) and 57 years (range, 31–88), respectively. Both sample groups have a similar male and Caucasian predominance. Pretreatment ($n = 71$) and normal screening salivary ($n = 61$) rinses cohort had similar sample sizes. Similarly, the patients from whom the normal screening salivary rinses were obtained were distinctly younger. The male and Caucasian predominance was less pronounced in the normal salivary rinse samples, and there was larger proportion of nonsmokers (47% *vs*. 31%) and fewer current smokers (18% *vs*. 37%) within this group. Alcohol consumption of patients ($n = 71$) and control population ($n = 61$) in salivary rinse cohort was found 25% *vs*. 18% for past alcohol consumers and 34% *vs*. 49% for current consumers, respectively. For nonalcoholic group, the rates were similar as 23% for controls and 20% for patients.

Tumor patients were composed of those without tobacco history (33%), current smokers (31%) and those who had quit smoking (29%). Alcohol consumption in patients with tumor samples was found 20% for nonalcoholic patients, 20% for past alcohol consumers and 38% for current consumers. Tumor samples ($n = 101$) were obtained from patients with Stage I (18%), Stage II (16%), Stage III (12%), and Stage IV (54%) lesions at the time of presentation. These were from primary tumors of the oral cavity ($n = 39$), oropharynx ($n = 38$), hypopharynx ($n = 5$), larynx ($n = 15$), maxillary sinus ($n = 2$), and unknown primary/neck ($n = 2$). Pretreatment salivary rinses of cancer patients have similar characteristics as they were obtained from a subset ($n = 71$) of the same tumor patient population.

Initial screening of candidate genes

We aimed to evaluate the epigenetic changes specific to HNSCC by investigating promoter hypermethylation of a panel of 10 genes that has been previously published to be highly methylated in other tumor types but not in normal tissues, via candidate gene approach,[18–21, 23] by using Q-MSP. The promoter regions of *CDH4* (30%), *TERT* (19.6%), *MAL* (95%), *FKBP4* (38%), *VGF* (95%), *NISCH* (100%), and *PAK3* (78%) genes were mostly observed to be heavily methylated in normal salivary rinse samples, whereas only 3 genes from our candidate gene group, *KIF1A* (2%), *EDNRB* (6.6%), and *CD44* (4%), demonstrated a low rate and proportion of methylation (Fig. 1).

We selected these 3 genes to investigate the presence and level of gene promoter methylation in 33 tumor specimens from patients in a limited cohort. We observed 88% (29/33) and 84.8% (28/33) methylation rates for *KIF1A* and *EDNRB*, respectively, in primary HNSCC, whereas no methylation in the promoter region of *CD44* gene was observed (data not shown). In a further study, we expanded the patient cohort from the original 33 tumors, to include 101 tumors

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and 61 normals and showed a difference of methylation levels between normal salivary rinses and tumor samples for both *EDNRB* and *KIF1A*. Normal salivary rinses demonstrated minimal methylation levels (Fig. 2).

Validation of HNSCC specific methylation in tissue samples (mucosa vs. tumor)

The methylation status of 2 selected genes (*EDNRB* and *KIF1A*) were analyzed in 101 fresh tumors from a HNSCC patient cohort and normal mucosal samples from 15 healthy individuals (Fig. 3). *KIF1A* and *EDNRB* promoters were frequently methylated in both tumor and normal mucosa samples, with the normal mucosa samples exhibiting very low levels of methylation when compared to tumor samples as seen in the significantly different mean and median methylation values for each cohort. *KIF1A* was methylated in 98% [99/101, mean ± sd = 18.57 ± 2.53, median (range) = 5.02 (0, 100)] and *EDNRB* in 97% [98/101, mean ± sd = 27 ± 3.28, median (range) = 10.4 (0, 114.9)] of tumor samples. Only 18 out of 99 (18%) and 13% (13/99) of methylated tumor samples were observed to have equal to or less than 1 methylated copy in the promoter region of KIF1A and EDNRB gene, respectively. This may be due to low level methylation from a minor clonal population present in the sampled tumor specimen.

In normal mucosa samples, *KIF1A* was methylated in 86.6% [13/15, mean ± sd = 1.76 ± 0.62, median (range) = 0.66 (0, 9.22)] and EDNRB in 93% [14/15, mean ± sd = 1.55 ± 0.58, median (range) = 0.49 (0, 8.42)] of samples, but at very low levels. Both *KIF1A* ($p = 0.0004$, Wilcoxon rank test) and *EDNRB* ($p < 0.0001$, Wilcoxon rank test) have higher methylation levels in tumor than normal mucosa (Fig. 3).

Methylation in salivary rinses from HNSCC patients and healthy control subjects

The methylation status of 2 selected genes (*EDNRB* and *KIF1A*) were analyzed in 71 salivary rinses of patients with head and neck cancer and 61 salivary rinses from healthy individuals (Fig. 4). The specificity and sensitivity values showing the power of our candidate markers were given in Table 2.

In salivary rinse samples collected from patients before any treatment, *KIF1A* was methylated in 38% [27/71, mean ± sd = 1.76 ± 0.62, median (range) = 0 (0, 3.96)] and *EDNRB* in 67.6% [48/71, mean ± sd = 5.48 ± 2.18, median (range) = 0.06 (0, 120.5)] of samples whereas in salivary rinses from healthy people, these genes were methylated infrequently, 2% [1/47 (missing n = 14), mean ± sd = 0.1835 ± 0.072, median (range) = 0 (0, 0.12)] for *KIF1A* and 6.6% [3/45 (missing $n = 16$), mean ± sd = 0.0123 ± 0.007, median (range) = 0 (0, 0.2)] for *EDNRB* ($p < 0.0001$, Wilcoxon rank test of methylation levels for both genes). We also checked the correlation between *KIF1A* and *EDNRB* methylation levels. In tumor samples ($n = 101$), *KIF1A* and *EDNRB* were positively correlated ($r = 0.33$, $p = 0.0007$) but in pretreatment salivary rinse samples ($n = 71$) no significant correlation between *KIF1A* and *EDNRB* was seen ($p > 0.3$). Using data from 71 matched HNSCC patients and Wilcoxon rank test, we did not find significant difference in primary tumor methylation levels between patients with elevated saliva methylation level and patients without elevated saliva methylation level (all p-values > 0.20).

Correlation with clinical characteristics

We have sought correlation between the methylation status of these 2 genes in primary tumor samples with clinical parameters of HNSCC patients ($n = 101$). We found a significant association between *KIF1A* levels and tumor site (Table 3). Patients with tumor in oral cavity (n = 39), larynx ($n = 15$) and maxillary sinus ($n = 2$) had significantly higher *KIF1A* methylation level (mean ± sd; 27.0 ± 4.0) than patients with other tumor sites ($n = 45$, mean ± sd; 8.4 ± 2.0, $p = 0.0009$). We detected methylation in 8 of 27 (30%) and 19 out of 48 (39.5%) methylated salivary rinse samples from HNSCC patients having Stage I and II in the promoter region of

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KIF1A and EDNRB genes respectively. There was no association between the methylation levels and advanced stage or early stage tumors.

There was no significant association between *KIF1A* methylation level in HNSCC patients' salivary rinses and other clinical features, although 48 patients who were current or former smokers had lower *EDNRB* levels (mean ± sd; 2.3 ± 1.1) than 22 nonsmokers (mean ± sd;12.6 ± 6.4, p = 0.0266, Wilcoxon rank test). To verify whether this association is confounded with age, we performed 2 separated logistic regression analyses using saliva samples from 71 HNSCC patients and 61 normal controls: for KIF1A and EDNRB. From both models, we did not find any significant age effect (all p-values are greather than 0.10) (Table S3). No significant difference in mortality was found between patients with methylated *KIF1A* or *EDNRB* salivary rinses and patients without *KIF1A* and *EDNRB* methylation.

For HNSCC patients, both in primary tumors and salivary rinses, poorer survival was associated with age (Tables S4,S5), overall stage and tumor site in univariate analysis, and all 3 factors remained significant on multivariate analysis, but there was no association with survival and *KIF1A* and/or *EDNRB* methylation status and clinical outcome (Table S5).

Supplementary Figures 1–3 show survival analysis by overall stage, T stage and T stage and age in patients with HNSCC, respectively. Higher cancer overall stage was associated with poorer survival. In particular, stage 4 patients had significant higher mortality rate than other patients (RR = 2.65, $p = 0.061$). After adjusting for age at last follow up, tumor recurrent status, gender, alcohol use, and smoking, the risk ratio was RR = 4.093 ($p = 0.031$) (Figure S1). Patients with having stage T3 or T4 had a higher mortality rate than subjects with T stage <3 (RR = 4.37, $p = 0.0015$, Figure S2). However, for patients with stage T3 or T4, patients with age >62 had much higher mortality rate than patients with age < 62 ($p = 0.0004$, Figure S3).

Discussion

We evaluated the utility of detection of aberrant promoter methylation of these genes in the salivary rinses from cancer patients compared with normal population as a detection tool. Methylation of the *KIF1A* and *EDNRB* gene promoters is a frequent event in HNSCC with these genes being infrequently and minimally methylated in normal salivary rinses, demonstrating potential for these genes as biomarkers in detection strategies. To obtain an accurate determination of methylation status in a cohort of normal individuals, we chose to assess the presence of methylated signal in exfoliated upper aerodigestive cells obtained during a screening study in a control population. The sample collected using this technique includes exfoliated epithelial cells from the upper aerodigestive tract obtained during a rinse and gargle, and cells from the deep epithelial layers from the oral cavity and oropharynx by using the exfoliating brush. This technique allows for a broad sampling of epithelial cells from multiple sites in the upper aerodigestive tract.

KIF1A (Kinesin family member 1A) encodes a protein that is microtubule-dependent molecular motor involved in important intracellular functions such as organelle transport and cell division.[24] This protein is highly similar to mouse heavy chain kinesin member 1A protein, which is an anterograde motor protein that transports membranous organelles along axonal microtubules. It is thought that this protein may play a critical role in the development of axonal neuropathies resulting from impaired axonal transport.25

EDNRB (Endothelin receptor type B) is a G protein-coupled receptor, which activates a phosphatidylinositolcalcium second messenger system. Its ligand, endothelin, consists of a family of 3 potent vasoactive peptides: ET1, ET2, and ET3. Studies suggest that the multigenic disorder, Hirschsprung disease type 2, is due to mutation in endothelin receptor type B gene.[26]

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There are only 2 previous publications from our group, showing the methylation status of *KIF1A* gene in human cancers.[18,21] The promoter methylation status of *EDNRB* gene was studied previously in bladder,[27,28] renal,[29] prostate,[19,30] lung,[31] medullablastoma,[32] and hepatocellular carcinoma.[33] Two reports have been published focusing on EDNRB methylation in nasopharyngeal carcinoma[34,35] but none involving the other head and neck sites.

In our study, methylation levels of both genes were very low in tissue specimens from healthy normal individuals even though a majority of these samples revealed a certain level of methylation in *EDNRB* or *KIF1A* gene promoters (>13 individuals from the cohort of 15). However, when analyzed in salivary rinses from healthy individuals, methylation of these 2 genes was not frequent (1/47, 2% for *KIF1A* and 3/45, 6.6% for *EDNRB*). Moreover, markedly increased levels of methylation in pretreatment salivary rinses from HNSCC patients strongly demonstrates the potential of measuring methylation levels in salivary rinse samples as a noninvasive approach for detection of HNSCC. We were able to show that *KIF1A* (97.8% specificity and 36.6% sensitivity) and EDNRB (93.2% specificity and 67.6% sensitivity) are highly sensitive markers that could potentially be used for molecular detection strategies. In addition, combining the markers improves sensitivity while maintains good specificity (93.1% specificity and 77.4% sensitivity). We have previously reported[7] that using a panel of 4 different genes (MINT31, MGMT, CCNA1, p16), methylation detection sensitivity in salivary rinses of HNSCC patients improved to 35% without significantly compromising the specificity (90%). We were interested in defining new genes that would be of utility in molecular detection strategies, hence we evaluated a panel of 10 additional genes that are frequently methylated in other tumor types as has been previously reported in the literature and so were considered separately from our previously reported cohort. This study demonstrates an increase of sensitivity with similar specificity using a panel of only 2 genes. The methylation detection of these 2 genes has potential to be developed as a noninvasive tool for HNSCC detection screening. Promoter hypermethylation patterns in individual tumors show variation depending on specific altered molecular pathways and so it is anticipated that the use of more than 1 gene will provide greater applicability and coverage for diverse tumors when compared with a single gene for cancer detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. CA Cancer J Clin 2006;56:106–130. [PubMed: 16514137]
2. Palmisano WA, Divine KK, Saccomanno G, Gilliland FD, Baylin SB, Herman JG, Belinsky SA. Predicting lung cancer by detecting aberrant promoter methylationinsputum. Cancer Res 2000;60:5954–5958. [PubMed: 11085511]
3. Hoque MO, Begum S, Topaloglu O, Jeronimo C, Mambo E, Westra WH, Califano JA, Sidransky D. Quantitative detection of promoter hypermethylation of multiple genes in the tumor, urine, and serum DNA of patients with renal cancer. Cancer Res 2004;64:5511–5517. [PubMed: 15289362]
4. Nunes DN, Kowalski LP, Simpson AJ. Detection of oral and oropharyngeal cancer by microsatellite analysis in mouth washes and lesion brushings. Oral Oncol 2000;36:525–528. [PubMed: 11036246]

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5. Rosas SL, Koch W, da Costa Carvalho MG, Wu L, Califano J, Westra W, Jen J, Sidransky D. Promoter hypermethylation patterns of p16, O6-methylguanine-DNAmethyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. Cancer Res 2001;61:939–942. [PubMed: 11221887]

6. El-Naggar AK, Mao L, Staerkel G, Coombes MM, Tucker SL, Luna MA, Clayman GL, Lippman S, Goepfert H. Genetic heterogeneity in saliva from patients with oral squamous carcinomas: implications in molecular diagnosis and screening. J Mol Diagn 2001;3:164–170. [PubMed: 11687600]

7. Carvalho AL, Jeronimo C, Kim MM, Henrique R, Zhang Z, Hoque MO, Chang S, Brait M, Nayak CS, Jiang WW, Claybourne Q, Tokumaru Y, et al. Evaluation of promoter hypermethylation detection in body fluids as a screening/diagnosis tool for head and neck squamous cell carcinoma. Clin Cancer Res 2008;14:97–107. [PubMed: 18172258]

8. Lee A, Kim Y, Han K, Kang CS, Jeon HM, Shim SI. Detection of tumor markers including carcinoembryonic antigen, APC, and cyclin D2 in fine-needle aspiration fluid of breast. Arch Pathol Lab Med 2004;128:1251–1256.

9. Usadel H, Brabender J, Danenberg KD, Jerónimo C, Harden S, Engles J, Danenberg PV, Yang S, Sidransky D. Quantitative adenomatous polyposis coli promoter methylation analysis in tumor tissue, serum, and plasma DNA of patients with lung cancer. Cancer Res 2002;62:371–375. [PubMed: 11809682]

10. Hu S, Ewertz M, Tufano RP, Brait M, Carvalho AL, Liu D, Tufaro AP, Basaria S, Cooper DS, Sidransky D, Ladenson PW, Xing M. Detection of serum deoxyribonucleic acid methylation markers: a novel diagnostic tool for thyroid cancer. J Clin Endocrinol Metab 2006;91:98–104. [PubMed: 16263813]

11. Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, Jen J, Herman JG, Sidransky D. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res 2000;60:892–895. [PubMed: 10706101]

12. Wong IH, Zhang J, Lai PB, Lau WY, Lo YM. Quantitative analysis of tumor-derived methylated p16INK4a sequences in plasma, serum, and blood cells of hepatocellular carcinoma patients. Clin Cancer Res 2003;9:1047–1052. [PubMed: 12631605]

13. Wadsworth JT, Somers KD, Stack BC Jr, Cazares L, Malik G, Adam BL, Wright GL Jr, Semmes OJ. Identification of patients with head and neck cancer using serum protein profiles. Arch Otolaryngol Head Neck Surg 2004;130:98–104. [PubMed: 14732777]

14. Bernard PS, Wittwer CT. Real-time PCR technology for cancer diagnostics. Clin Chem 2002;48:1178–1185. [PubMed: 12142370]

15. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000;28:E32. [PubMed: 10734209]

16. Cottrell SE, Laird PW. Sensitive detection of DNA methylation. Ann N Y Acad Sci 2003;983:120–130. [PubMed: 12724217]

17. Jerónimo C, Usadel H, Henrique R, Oliveira J, Lopes C, Nelson WG, Sidransky D. Quantitation of GSTP1methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. J Natl Cancer Inst 2001;93:1747–1752. [PubMed: 11717336]

18. Hoque MO, Kim MS, Ostrow KL, Liu J, Wisman GB, Park HL, Poeta ML, Jeronimo C, Henrique R, Lendvai A, Schuuring E, Begum S, et al. Genome-wide promoter analysis uncovers portions of the cancer methylome. Cancer Res 2008;68:2661–2670. [PubMed: 18413733]

19. Woodson K, Gillespie J, Hanson J, Emmert-Buck M, Phillips JM, Linehan WM, Tangrea JA. Heterogeneous gene methylation patterns among pre-invasive and cancerous lesions of the prostate: a histopathologic study of whole mount prostate specimens. Prostate 2004;60:25–31. [PubMed: 15129426]

20. Pu RT, Sheng ZM, Michael CW, Rhode MG, Clark DP, O'Leary TJ. Methylation profiling of mesothelioma using real-time methylation-specific PCR: a pilot study. Diagn Cytopathol 2007;35:498–502. [PubMed: 17636483]

22. Ostrow KL, Park HL, Hoque MO, Kim MS, Liu J, Argani P, Westra W, Van Criekinge W, Sidransky D. Pharmacologic unmasking of epigenetically silenced genes in breast cancer. Clin Cancer Res 2009;15:1184–1191. [PubMed: 19228724]

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22. Park HL, Kim MS, Yamashita K, Westra W, Carvalho AL, Lee J, Jiang WW, Baek JH, Liu J, Osada M, Moon CS, Califano JA, et al. DCC promoter hypermethylation in esophageal squamous cell carcinoma. Int J Cancer 2008;122:2498–2502. [PubMed: 18302152]

23. Miotto E, Sabbioni S, Veronese A, Calin GA, Gullini S, Liboni A, Gramantieri L, Bolondi L, Ferrazzi E, Gafà R, Lanza G, Negrini M. Frequent aberrant methylation of the CDH4 gene promoter in human colorectal and gastric cancer. Cancer Res 2004;64:8156–8159. [PubMed: 15548679]

24. Okada Y, Yamazaki H, Sekine-Aizawa Y, Hirokawa N. The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. Cell 1995;81:769–780. [PubMed: 7539720]

25. Yonekawa Y, Harada A, Okada Y, Funakoshi T, Kanai Y, Takei Y, Terada S, Noda T, Hirokawa N. Defect in synaptic vesicle precursor transport and neuronal cell death in KIF1A motor protein-deficient mice. J Cell Biol 1998;141:431–441. [PubMed: 9548721]

26. Smollich M, Wülfing P. Targeting the endothelin system: novel therapeutic options in gynecological, urological and breast cancers. Expert Rev Anticancer Ther 2008;8:1481–1493. [PubMed: 18759699]

27. Yates DR, Rehman I, Abbod MF, Meuth M, Cross SS, Linkens DA, Hamdy FC, Catto JW. Promoter hypermethylation identifies progression risk in bladder cancer. Clin Cancer Res 2007;13:2046–2053. [PubMed: 17404085]

28. Friedrich MG, Chandrasoma S, Siegmund KD, Weisenberger DJ, Cheng JC, Toma MI, Huland H, Jones PA, Liang G. Prognostic relevance of methylation markers in patients with non-muscle invasive bladder carcinoma. Eur J Cancer 2005;41:2769–2778. [PubMed: 16242928]

29. Yao M, Huang Y, Shioi K, Hattori K, Murakami T, Sano F, Baba M, Kondo K, Sano F, Baba M, Kondo K, Nakaigawa N, Kishida T, Nagashima Y, Yamada-Okabe H, et al. A three-gene expression signature model to predict clinical outcome of clear cell renal carcinoma. Int J Cancer 2008;123:1126–1132. [PubMed: 18546273]

30. Rogers CG, Gonzalgo ML, Yan G, Bastian PJ, Chan DY, Nelson WG, Pavlovich CP. High concordance of gene methylation in post-digital rectal examination and postbiopsy urine samples for prostate cancer detection. J Urol 2006;176:2280–2284. [PubMed: 17070312]

31. Chen SC, Lin CY, Chen YH, Fang HY, Cheng CY, Chang CW, Chen RA, Tai HL, Lee CH, Chou MC, Lin TS, Hsu LS. Aberrant promoter methylation of EDNRB in lung cancer in Taiwan. Oncol Rep 2006;15:167–172. [PubMed: 16328051]

32. Lindsey JC, Lusher ME, Anderton JA, Bailey S, Gilbertson RJ, Pearson AD, Ellison DW, Clifford SC. Identification of tumour-specific epigenetic events in medulloblastoma development by hypermethylation profiling. Carcinogenesis 2004;25:661–668. [PubMed: 14688019]

33. Hsu LS, Lee HC, Chau GY, Yin PH, Chi CW, Lui WY. Aberrant methylation of EDNRB and p16 genes in hepatocellular carcinoma (HCC) in Taiwan. Oncol Rep 2006;15:507–511. [PubMed: 16391877]

34. Lo KW, Tsang YS, Kwong J, To KF, Teo PM, Huang DP. Promoter hypermethylation of the EDNRB gene in nasopharyngeal carcinoma. Int J Cancer 2002;98:651–655. [PubMed: 11920632]

35. Zhou L, Feng X, Shan W, Zhou W, Liu W, Wang L, Zhu B, Yi H, Yao K, Ren C. Epigenetic and genetic alterations of the EDNRB gene in nasopharyngeal carcinoma. Oncology 2007;72:357–363. [PubMed: 18187958]

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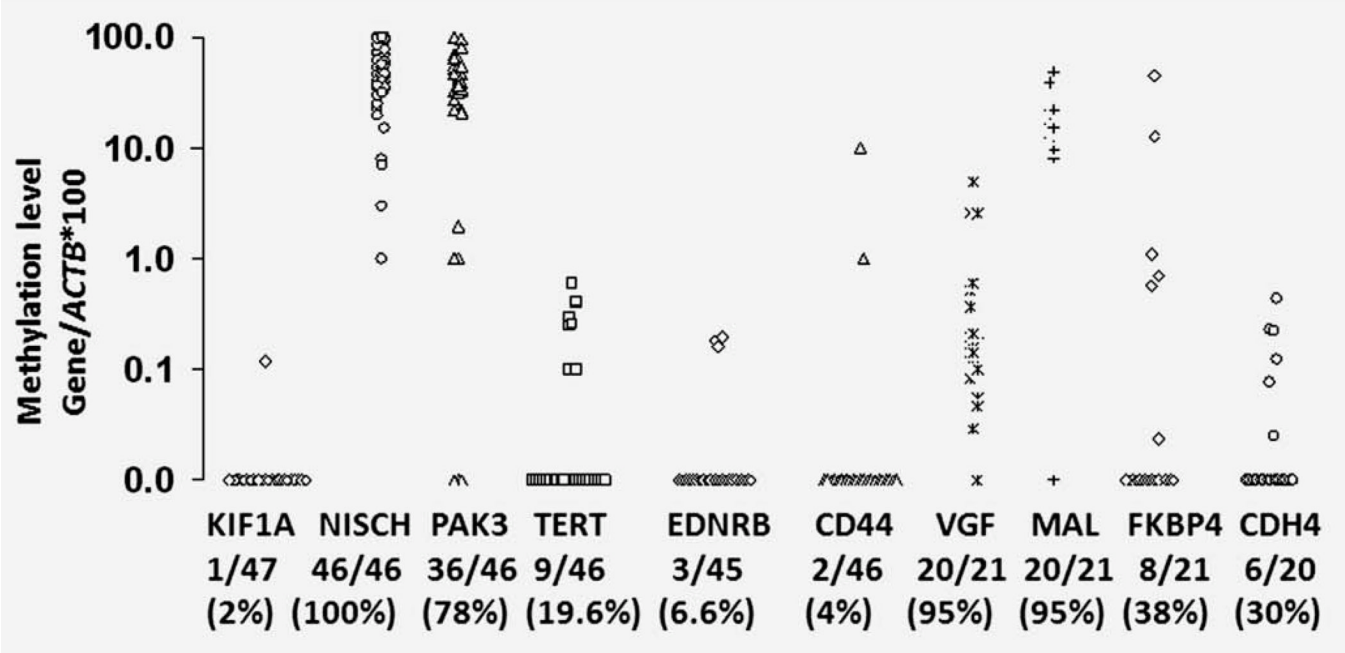

Figure 1.
The relative methylation frequencies of candidate genes in normal salivary samples. Scatter plots of QMSP analysis of candidate gene promoters. X axis, proportion of methylated cases/tested cases for each gene; Y axis, quantity of hypermethylation (gene of interest/ACTB×100).

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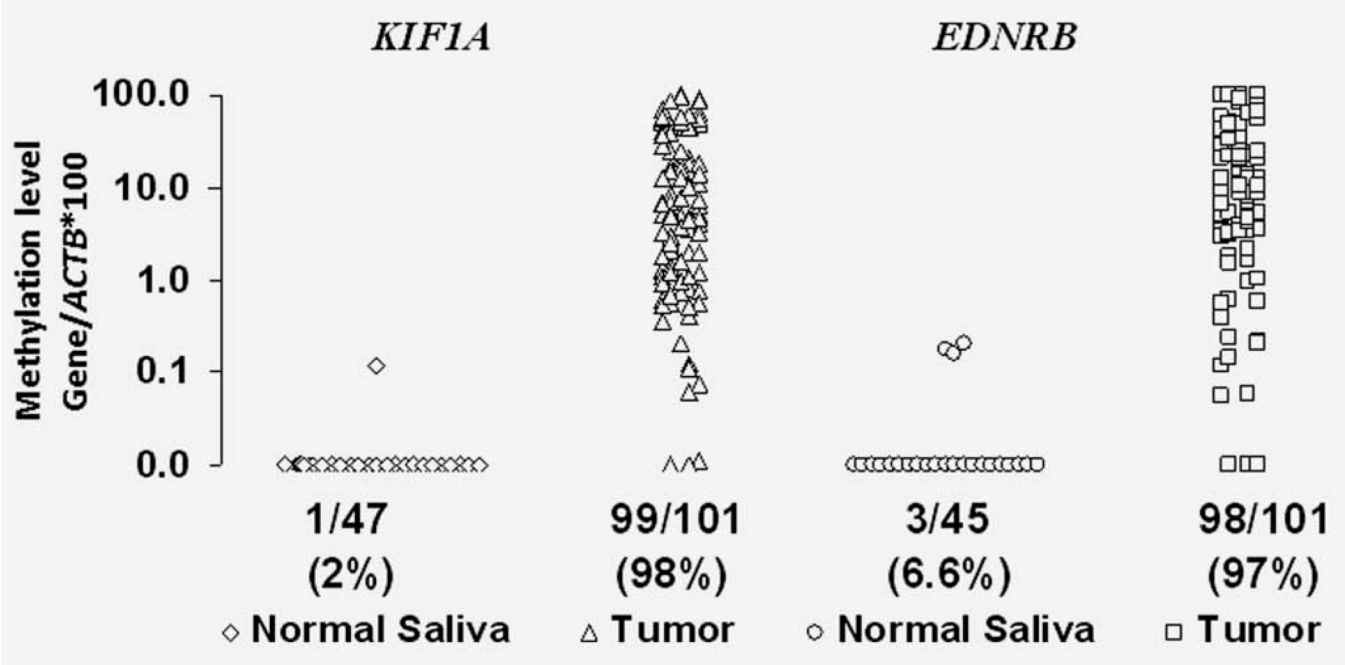

Figure 2.
Patterns of hypermethylation of *KIF1A* and *EDNRB* genes observed in DNA from tumor samples of patients with HNSCC and salivary rinses from healthy population. X axis, proportion of methylated cases/tested cases for each sample type; Y axis, quantity of hypermethylation (gene of interest/ACTB×100).

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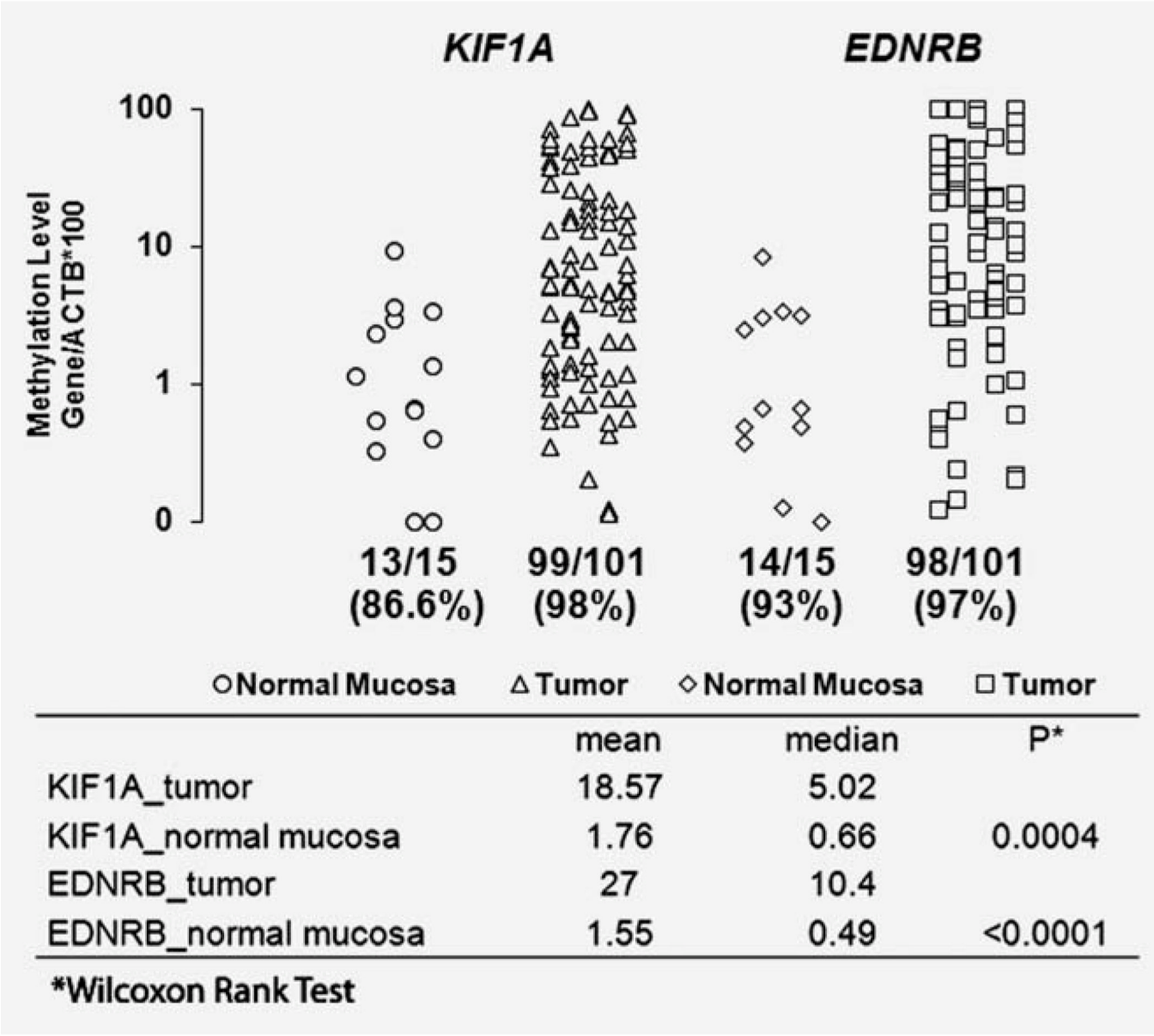

	mean	median	P*
KIF1A_tumor	18.57	5.02	
KIF1A_normal mucosa	1.76	0.66	0.0004
EDNRB_tumor	27	10.4	
EDNRB_normal mucosa	1.55	0.49	<0.0001

*Wilcoxon Rank Test

Figure 3.

The methylation rates on *KIF1A* and *EDNRB* in 101 fresh tumor samples of patients with HNSCC and 15 normal mucosa from healthy individuals. X axis, proportion of methylated cases/tested cases for each sample type; Y axis, quantity of hypermethylation (gene of interest/ACTB×100).

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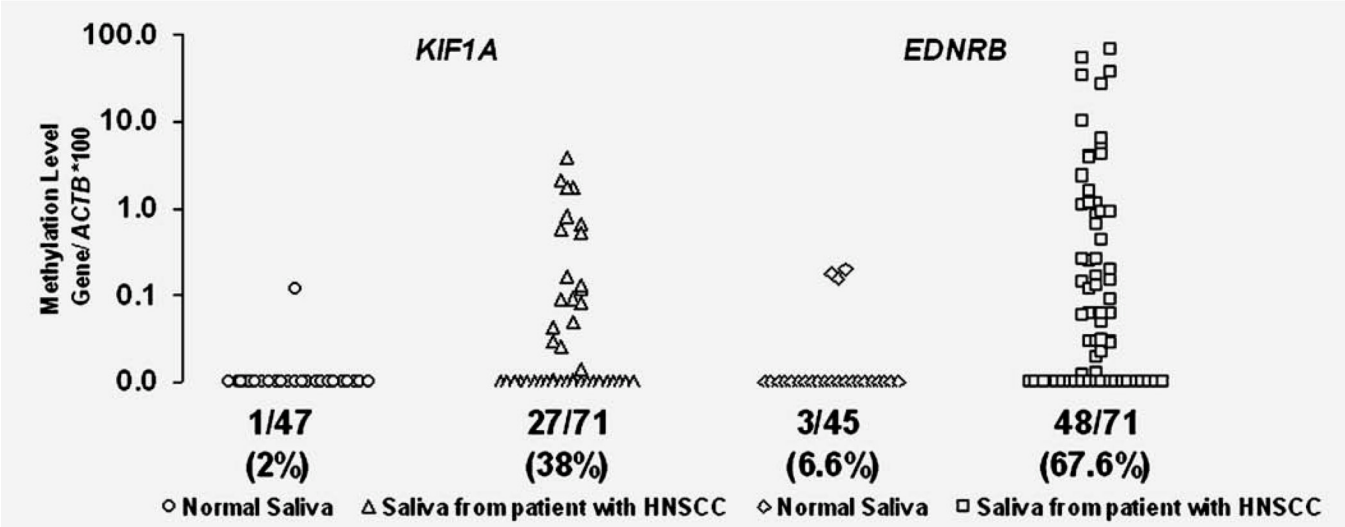

	mean	median	P*
KIF1A_HNSCC salivary rinses	1.76	0	<0.0001
KIF1A_normal salivary rinses	0.18	0	
EDNRB_HNSCC salivary rinses	5.48	0.06	<0.0001
EDNRB_normal salivary rinses	0.012	0	

*Wilcoxon Rank Test

Figure 4.

Patterns of hypermethylation on *KIF1A* and *EDNRB* in 71 salivary rinses from patients with HNSCC collected before any treatment and 61 normal salivary rinse samples from healthy individuals. X axis, proportion of methylated cases/tested cases for each sample type; Y axis, quantity of hypermethylation (gene of interest/ACTB×100).

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Table 1

Demographics of study patients

	Head and neck cancer tumor samples (*N* = 101)	Normal mucosa samples (*N* = 15)	Head and neck cancer salivary rinse samples (*N* = 71)	Normal salivary rinse samples (*N* = 61)
Age				
Mean	56.9 ± 12.9 (31–88)	43.2 ± 14.6 (22–65)	57.2 ± 12.0 (31–87)	51.8 ± 14.3 (19–80)
Sex				
Male	78 (77%)	10 (67%)	55 (77%)	36 (59%)
Female	23 (23%)	3 (20%)	16 (23%)	25 (41%)
Race				
Caucasian	86 (85%)	10 (61%)	61 (86%)	37 (61%)
Black	12 (12%)	?	8 (11%)	21 (34%)
Other	3 (3%)	1 (7%)	2 (3%)	3 (5%)
Smoking status				
Never	32 (33%)	9 (60%)	22 (31%)	29 (47%)
Former	30 (29%)	5 (33%)	22 (31%)	20 (33%)
Current	32 (18%)	0 (0%)	26 (37%)	11 (18%)
Unknown	7 (7%)	1 (7%)	1 (1%)	1 (2%)
Alcohol status				
Never	20 (20%)	10 (66%)	14 (20%)	14 (23%)
Former	20 (20%)	1 (7%)	18 (25%)	11 (18%)
Current	39 (38%)	3 (20%)	24 (34%)	30 (49%)
Unknown	22 (22%)	1 (7%)	15 (21%)	6 (10%)
Stage				
I	18 (18%)	-	13 (18%)	-
II	16 (16%)	-	10 (14%)	-
III	12 (12%)	-	10 (14%)	-
IV	55 (54%)	-	38 (54%)	-
Site				
Oral cavity	39 (38%)	-	19 (27%)	-
Oropharynx	38 (38%)	-	30 (42%)	-
Hypopharynx	5 (5%)	-	5 (7%)	-
Larynx	15 (15%)	-	13 (18%)	-
Neck/other	2 (2%)	-	2 (3%)	-
Maxillary sinus	2 (2%)	-	2 (3%)	-

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Table 2

Comparison of hypermethylation detection on salivary rinses from patients with HNSCC and healthy control samples

Genes	Sensitivity,[1] % (95% Cl)	Specificity,[1] % (95% Cl)
KIF1A	36.60 (22.70–50.50)	97.80 (94.40–100)
EDNRB	67.60 (53.80–81.40)	93.20 (87.30–99.0)
KIF1A + *EDNRB*	77.40 (62.20–92.70)	93.10 (87.20–99.0)

[1]The cutoff point is zero for both *KIF1A* and *EDNRB*.

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Table 3

Percentiles and mean values of KIF1A methylation in HNSCC primary tumor samples at different tumor sites

Tumor site	25th percentile	50th percentile	75th percentile	Mean	Standard error
Oral cavity	4.52	14.72	49.04	27.97	4.93
Oropharynx	1.32	3.2	7.09	9.53	2.36
Hypopharynx	0.71	0.78	0.91	2.33	1.57
Larynx	1.17	6.7	48.42	25.19	7.9
Maxillary sinus	21.98	22.84	23.71	22.84	1.73
Neck/other	2.25	2.7	3.15	2.7	0.9

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