International Journal of Cancer

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Journal:	*International Journal of Cancer*
Manuscript ID:	IJC-09-2307
Wiley - Manuscript type:	Early Detection and Diagnosis
Date Submitted by the Author:	28-Sep-2009
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Key Words:	Methylation, Saliva, head and neck cancer, KIF1A, EDNRB

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

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Financial Disclosures: No conflicts of interest exist.

Keywords: Methylation, saliva, head and neck cancer, *KIF1A*, *EDNRB*

Running title: Methylation of *KIF1A* and *EDNRB* in Saliva of HNSCC patients.

Abbreviations:

EDNRB: endothelin receptor type B; *KIF1A*: kinesin chain member 1A, HNSCC: head and neck squamous-cell carcinoma; Q-MSP: quantitative methylation-specific PCR.

Statement of Translational Relevance

DNA methylation biomarkers detection in salivary rinse samples have great potential to be developed as a non-invasive tool for head and neck cancer early diagnosis. We have previously reported frequent methylation of another panel of methylation markers in salivary rinses of patients with head and neck cancer. In this study, methylation of two genes, *KIF1A* and *EDNRB*, were shown to be methylated primary head and neck cancer, and highly specific markers for head and neck cancer in salivary rinses from head and neck cancer patients.

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*) 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 two selected genes (*EDNRB* and *KIF1A*) were then analyzed in fifteen 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 prior to 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.

INTRODUCTION

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 thirty years despite active clinical and basic science research adressing 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 ten 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.

MATERIALS 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. 101 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 two 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 supernatant was discarded and DNA was isolated from the pellet. Tumors were snap frozen and microdissected on a cryostat to at least 75% purity. 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 two 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 (QMSP)

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 x 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; 1X Rox Dye reference and 1X 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). Three microliters of bisulfite treated DNA were 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 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. Pre-treatment (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 non-smokers (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 non-alcoholic 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 non-alcoholic 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). Pre-treatment 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 ten 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 three genes from our candidate gene group, *KIF1A* (2%), *EDNRB* (6.6%) and *CD44* (4%), demonstrated a low rate and proportion of methylation (Figure 1).

We selected these three 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 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 (Figure 2).

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

The methylation status of two selected genes (*EDNRB* and *KIF1A*) were analyzed in 101 fresh tumors from a HNSCC patient cohort and normal mucosal samples from 15 healthy individuals (Figure 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. 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 (Figure 3).

Methylation in Salivary rinses from HNSCC patients and healthy control subjects

The methylation status of two 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 (Figure 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 pre-treatment salivary rinse samples (n=71) no significant correlation between *KIF1A* and *EDNRB* was seen ($p>0.3$).

Correlation with Clinical Characteristics

We have sought correlation between the methylation status of these two 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 S3). 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, $p=0.0009$).

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 non-smokers (mean±sd;12.6±6.4, $p=0.0266$, Wilcoxon rank test). 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 three 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, Figures S1-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 phosphatidylinositol-calcium second messenger system. Its ligand, endothelin, consists of a family of three 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].

There are only two 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 two genes was not frequent (1/47, 2% for *KIF1A* and 3/45, 6.6% for *EDNRB*). Moreover, markedly increased levels of methylation in pre-treatment salivary rinses from HNSCC patients strongly demonstrates the potential of measuring methylation levels in salivary rinse samples as a non-invasive 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 two genes. The methylation detection of these two genes has potential to be developed as a non-invasive 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 one gene will provide greater applicability and coverage for diverse tumors when compared with a single gene for cancer detection.

ACKNOWLEDGEMENT

Dr. Califano is supported by a Clinical Innovator Award from the Flight Attendant Medical Research Institute.

This work was supported by a SPORE grant – P50 CA96784.

We would like to thank Steven Li (MS), Oluwasina Folawiyo (BS), Simy Buckwold (MS) and Andrew Chou (BS) for the technical help.

Disclosure of Potential Conflicts of Interest

Under a licensing agreement between Oncomethylome Sciences, SA and The Johns Hopkins University, D. Sidransky is entitled to a share of royalty received by the University upon sales of diagnostic products described in this article. 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.TheJohns Hopkins University in accordance with its conflict of interest policies is managing the terms of this agreement.

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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 (67%)	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 (31%)	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%)	-

256x232mm (72 x 72 DPI)

Table 2: Comparison of hypermethylation detection on salivary rinses from patients with HNSCC and healthy control samples.

Genes	*Sensitivity*, % (95% CI)*	*Specificity*, % (95% CI)*
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)**

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

224x82mm (72 x 72 DPI)

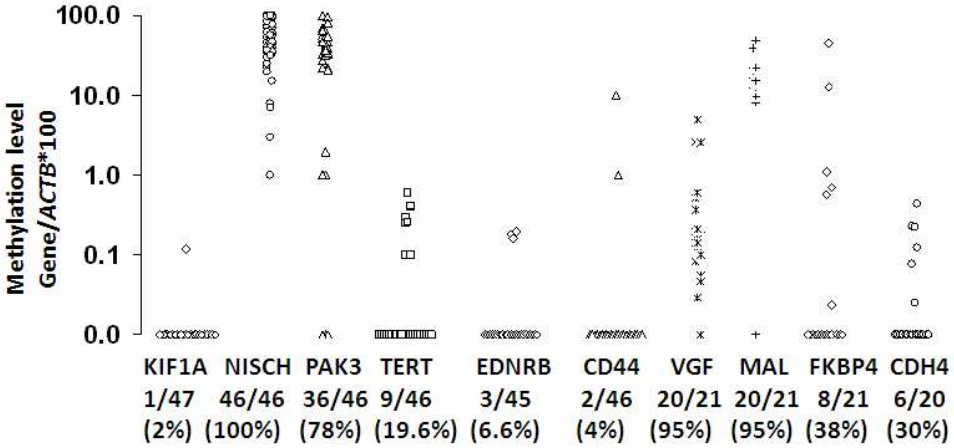


Figure 1: The relative methylation frequencies of candidate genes in normal salivary samples.
Scatter plots of QMSP analysis of candidate gene promoters.
X axis, proportion of methylated cases/tested cases for each gene;
Y axis, quantity of hypermethylation (gene of interest/ACTBx100).
283x153mm (72 x 72 DPI)

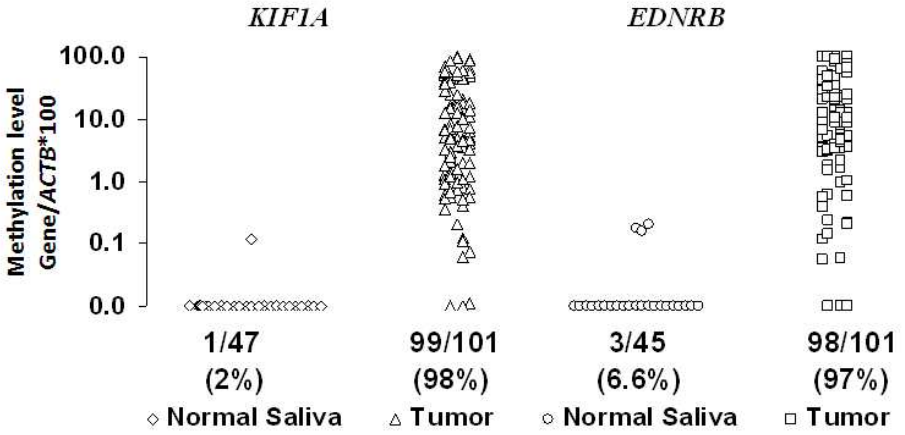
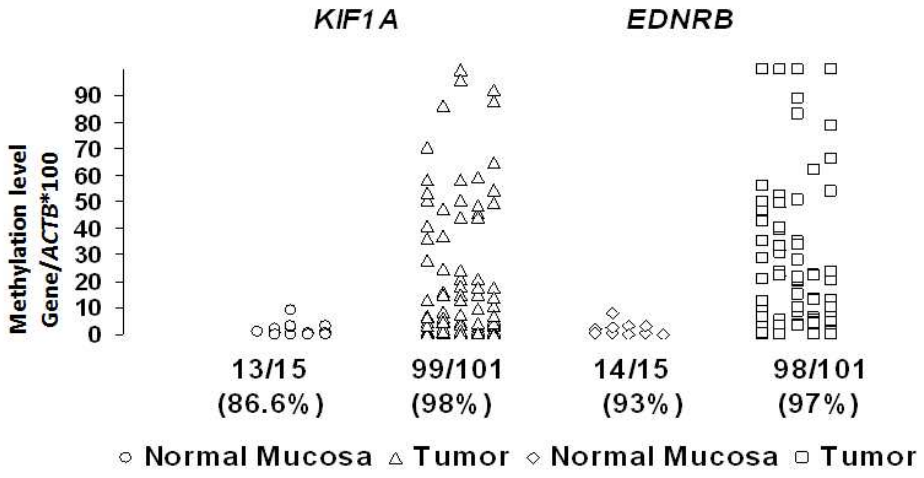


Figure 2: Patterns of hypermethylation of KIF1A and EDNRB genes observed in DNA from tumor samples of patients with HNSCC and salivary rinses from healthy population.
X axis, proportion of methylated cases/tested cases for each sample type;
Y axis, quantity of hypermethylation (gene of interest/ACTBx100).
288x143mm (72 x 72 DPI)



	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/ACTBx100).
287x240mm (72 x 72 DPI)

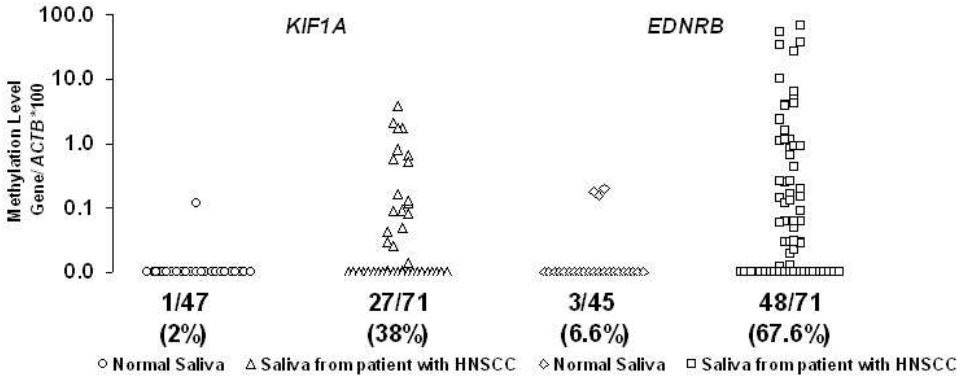


Figure 4: Patterns of hypermethylation on KIF1A and EDNRB in 71 salivary rinses from patients with HNSCC collected before any treatment and 61 normal salivary rinse samples from healthy individuals.
X axis, proportion of methylated cases/tested cases for each sample type;
Y axis, quantity of hypermethylation (gene of interest/ACTBx100).
272x130mm (72 x 72 DPI)