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Endothelin receptor type B gene promoter hypermethylation in salivary rinses independently associates with risk of oral cavity cancer and premalignancy

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Abstract

Endothelin receptor type B (*EDNRB*) and kinesin family member 1A(*KIF1A)* are candidate tumor suppressor genes that are inactivated in cancers. In this study we evaluated promoter

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hypermethylation of *EDNRB* and *KIF1A* and their potential use for risk classification in prospectively collected salivary rinses from patients with premalignant/malignant oral cavity lesions. Quantitative methylation-specific PCR(Q-MSP) was performed analyzing methylation status of *EDNRB* and *KIF1A* in salivary rinses of 191 patients. We proceeded to determine the association of methylation status with histologic diagnosis and estimate classification accuracy. On univariate analysis, diagnosis of dysplasia/cancer was associated with age and *KIF1A* or *EDNRB* methylation. Methylation of *EDNRB* highly correlated with that of *KIF1A*($p<0.0001$). On multivariable modeling, histologic diagnosis independently associated with *EDNRB*($p=0.0003$) or *KIF1A*($p=0.027$) methylation). A subset of patients analyzed (n=161) without prior biopsy proven malignancy received clinical risk classification based on examination. On univariate analysis, *EDNRB* and risk classification were associated with diagnosis of dysplasia/cancer, and remained significant on multivariate analysis (EDNRB:$p=0.047$, risk classification:$p=0.008$). Clinical risk classification identified dysplasia/cancer with a sensitivity of 71% and specificity of 58%. The sensitivity of clinical risk classification combined with *EDNRB* methylation improved to 75%.

EDNRB methylation in salivary rinses was independently associated with histologic diagnosis of premalignancy and malignancy and may have potential in classifying patients at risk for oral premalignant and malignant lesions in settings without access to a skilled dental practitioner. This may also potentially identify patients with premalignant and malignant lesions that do not meet criteria for high clinical risk based on skilled dental examination.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) accounts for greater than 37,000 new cases in the United States each year. The incidence of oral cavity cancer was approximated as 35,310 new cases per year and 7,590 estimated deaths.[1] Over the years, improvements have been made in the diagnosis, management, and targeted therapies for these cancers. However, despite these advances a considerable number of patients continue to present with advanced stage disease. Advanced staged cancers have traditionally been associated with higher rates of mortality and decreased locoregional control rates. Intuitively, early detection of oral cancers would lead to improved quality of life and survival for these patients. The cost-effectiveness for screening in oral cavity cancers in high-risk patients has been previously demonstrated.[2]

The application of salivary rinses from high-risk patients has been explored as a potential for molecular screening in HNSCC. [3–7] The inactivation of tumor suppressor genes caused by epigenetic changes such as promoter region CpG island hypermethylation has been well established in the literature.[8–10] The use of real time quantitative methylation-specific PCR (Q-MSP) provides a high throughput mechanism for detecting promoter hypermethylation in patient samples. The ability to quantify the methylation through Q-MSP allows for the potential of identifying high-risk patients with premalignant lesions. This has been previously demonstrated in salivary rinse samples obtained in lung cancer patients [11], oral cavity cancer patients [12] and oropharynx/hypopharynx cancer patients.[3]

We have previously published results of salivary rinse screening using promoter hypermethylation based markers in patients with previously diagnosed HNSCC.[12] To date, however, the effectiveness of this strategy which includes salivary rinse samples collected in a prospective cohort at who are at risk for oral cancer and oral premalignancy has not yet been evaluated. In this study, we evaluated the utility of detection of methylation of two gene promoters, *KIF1A* and *EDNRB*, and the association of methylation of these promoter regions with the presence of oral cancer and premalignancy.

METHODS

Tissue Samples

Salivary rinse samples from 191 patients were prospectively obtained from the dental and oral medicine clinics at New York University College of Dentistry (NYUCD) (140 patients, 73.30%), University of Puerto Rico (39 patients, 20.42%), St. Vincent's Cancer Center (1 patient, 0.52%), and Moffitt Cancer Center (11 patients, 5.76%). Institutional review board approval was obtained prior to the collection of the samples. A written informed consent was obtained from each subject. Enrollment included collection of demographic information and risk factor history (tobacco and alcohol). Inclusion criteria for enrollment were as follows: 1) English and/or Spanish speaking, 2) Age>18years, 3) the presence of a candidate oral epithelial lesion, and 4) the absence of a medical condition that would preclude a scalpel biopsy. Those lesions with an obvious etiology such as trauma, apthous ulceration, infection, or lichen planus were excluded. All patients were enrolled and assigned lesion risk classifications of low or high risk; this included an additional group of patients with histopathologically confirmed oral cancer who were also enrolled. Low risk groups were defined as having leukoplakia without the presence of erythroplakia, ulceration, erosion, or submucosal extension/induration. High risk patients exhibited one or more of following: leukoplakia with ulceration, erosion, or submucosal/extension; erythroplakia; erythroleukoplakia; or ulceration. If a subject presented with more than one lesion, each lesion was separately classified and the overall risk classification was designated based on the worst lesion.

Salivary rinses were obtained on all subjects as previously described.[12] In brief, salivary rinses were then obtained by rinsing and gargling with 25cc of normal saline solution for 15 seconds. Three strokes were performed using cotton tipped applicators to collect exfoliated cells from the buccal mucosa, alveolar ridge, lateral tongue, floor of mouth, and pharyngeal inlet. The cellular materials from the applicators were also agitated and released into the salivary rinse specimen. This was then centrifuged to obtain a cell pellet after discarding the supernatant. Pellets were then immediately frozen and stored at −80°C. All low and high risk lesions underwent an incisional scalpel biopsy and a histologic diagnosis was obtained which categorized the patients into one of six histologic diagnoses. Each specimen was examined by two calibrated oral pathologists blinded to the results of any other clinical data. Each specimen was examined at multiple levels and, in addition to a standard description and diagnosis, each pathologist recorded a possible primary histopathology outcome classification: benign (with or without atypia), dysplasia (mild, moderate and severe grades), and invasive carcinoma (squamous cell carcinoma). Differences between oral pathologist classifications were resolved by joint review resulting in a consensus classification.

The tissue samples and clinical data were obtained from NYUCD in a collaborative effort by the Department of Otolaryngology-Head and Neck Surgery at Johns Hopkins Medical Institutions, Baltimore. These patients constituted a subset of patients analyzed as part of a study previously conducted under a U54 mechanism at the NYUCD (U54DE014257, Dr. David Sirois). The following procedures, including DNA extraction, bisultfite treatment, and Q-MSP, were all performed by two individuals blinded to the clinical data pertaining to the clinical risk classification and the histologic diagnoses.

DNA extraction

DNA obtained from the salivary rinse samples was extracted by the tissue bank by digestion with 50μg/ml of proteinase K (Boehringer) in the presence of 1% SDS at 48°C overnight followed by phenol/chloroform extraction and ethanol precipitation.

Bisulfite treatment

The DNA obtained from the salivary rinse samples were subjected to bisulfite treatment as has been previously described.[13] Briefly, the EpiTect® Bisulfite Kit was used to for the conversion of 2μg of genomic DNA. The included Qiagen protocol was followed. After thermal denaturation and sodium bisulfite DNA conversion, the DNA was applied to an EpiTect spin plate. Optimized buffers and a vacuum manifold were used to wash and remove all traces of sodium bisulfate. The DNA was eluted. The eluted DNA was then ready for use for quantitative methylation-specific PCR.

Quantitative methylation-specific PCR

The bisulfite treated DNA was used as a template for flourescence based real-time QMSP as described previously.[14] The EDNRB and KIF1A genes had been previously detected on a prior screen of salivary rinses in HNSCC patients.[15] We had previously optimized the primer and probe sequences for Q-MSP. Briefly, primers and probes were designed specifically to amplify the bisulfite-converted DNA for the *βACTIN*, *EDNRB*, and *KIF1A* genes. *βACTIN* forward primer, 5′-TGGTGATGGAGG-AGGTTTAGTAAGT-3′, *βACTIN* reverse primer, 5′-AACCAATAAAACCTACTCCTCCCT-TAA-3′, and *βACTIN* TaqMan probe, 5′-ACCACCACCCAACACACAATAACAAACACA-3′. *EDNRB forward primer, 5′*-GGGAGTTGTAGTTTAGTTAGTTAGGGAGTAG-3′, *EDNRB* reverse primer, 5′-CCCGCGATTAAACTCGAAAA-3′, and *EDNRB* TaqMan probe, 5′-TTTTTATTCGTCGGGAGGAG-3′. *KIF1A* forward primer, 5′-GCGCGATAAATTAGTTGG-CGATT-3′, *KIF1A* reverse primer, 5′-CTCGACGACTACTCTACGCTAT-3′ and *KIF1A* TaqMan probe, 5′-CCTCCCGAAACGCTAATTAACTACGCG-3′. The ratios between the values of the *EDNRB* gene the reference gene *βACTIN* was obtained by TaqMan analysis and used as a measure for representing the relative quantity of methylation in a particular sample (value for gene of interest/value for *βACTIN* gene × 100). Flourogenic PCRs were carried out in a reaction volume of 10 μl 300nmol/L of each primer; 100nmol/L of probe; .375 unite of platinum Taq polymerase (Invitrogen); 100μmol/L of each dATP, dCTP, dGTP, and dTTP; 100nmol/L of ROX Reference Dye (Invitrogen); 8.4mmol/L ammonium sulfate; 33.5mmol/L Trizma (Sigma); 3.35 mmol/L magnesium chloride; 5mmol/L mercaptoethanol; and 0.05% DMSO. Each real time Q-MSP reaction consisted of 1.5μl of treated DNA solution. Amplifications were carried out in 384-well plates in a 7900 Sequence Detector System (Perkin-Elmer Applied Biosystems). Thermal cycling was initiated with a first denaturation step at 95°C for 2min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Each reaction was done in triplicate; the average of the triplicate was considered for analysis. The triplicate reactions also provided evidence of reproducibility of the individual reactions. Standardization was obtained by collecting leukocytes from a healthy individual that were subsequently methylated *in vitro* with excess Sss1 methyltransferase (New England Biolabs) to generate completely methylated DNA. This DNA was then Bisulfite treated as described above. Serial dilutions of this DNA were used for constructing the calibration curves on each plate. A separate sample of leukocytes from a healthy individual was obtained and only Bisulfite treatment was performed on the samples. These samples were used as a negative control for the reactions. There were also several control wells in each plate that contained only the reaction mix and water to ensure there was no contamination. Results for Q-MSP were analyzed considering the quantity of methylation (normalized by *βACTIN*) as well as considering the quantity of methylation as a binary event, in which any quantity of methylation in a sample would be considered positive for methylation.

Statistical Analysis

Proportions of *EDNRB* or *KIF1A* gene methylation were compared between patient salivary rinse samples. The initial analyses were performed with a cohort including 191 patients, where the subcategories of histologic outcome included benign, premalignant and malignant. Two pre-specified candidate genes (e.g., *EDNRB* and *KIF1A*) were evaluated. Gene hypermethylation was dichotomized at zero (i.e., no methylation vs. any methylation). Predictors associated with head and neck cancers were evaluated as well, including age, gender, race, smoking status, and alcohol consumption. Age was analyzed as a continuous variable, whereas all the other variables were considered as categorical variables. Univariate and multivariable proportional odds modeling were constructed sequentially to first explore the association of the variables of interest with the histologic outcome. Variables of significance based on the univariate models ($p<.20$) along with those deemed to be biologically/clinically important were retained for further analysis. Simultaneous effects expressed by these variables were studied using the multivariable proportional odds model. Odds ratios were reported with 95% confidence intervals, which indicated the strength of the association and its uncertainty. Throughout the analyses, proportional odds assumption of common slope for all of the cumulative logits was checked by Score test.

A secondary analysis explored the association of methylation that is independent of other predictors of histology, where patients with known cancer prior to coming to the clinic were excluded (n=30). This subset of 161 patients, i.e. the patient cohort not including the known cancer patients, was categorized as benign vs. dysplasia/cancer exploring the association of clinical risk classification to histopathology. Univariate and multivariable logistic regression analyses were performed using the same biologically/clinically important covariates as described above.

Receiver operating characteristic (ROC) analyses were conducted to estimate classification accuracy, sensitivity (true-positive rate) and specificity (false-positive rate) of the predictor along with 95% confidence intervals. AUC, an index of predictive power, was also provided. A logistic prediction model using the clinical risk classification combined with methylation of *EDNRB* was developed and internally validated. Statistical analyses were performed using SAS (v 9.2, SAS Institute, Cary, NC) and STATA software (v 8.2, College Station, Texas, and all statistical tests were two-sided with $p < 0.05$ considered statistically significant.

RESULTS

Population Characteristics

In analyzing the population characteristics of patients with oral cavity lesions (n=191) we found that the majority of patients were males and Caucasian. There were 68.1%, 67.4%, and 74.3% males in benign, dysplasia, and cancer categories respectively. We noted also noted that there were 68.1%, 74.4%, and 68.6% Caucasians in benign, dysplasia, and cancer categories respectively. Median age was 54 years and ranged from 18 to 90 years in the entire cohort. The baseline characteristics of the groups were similar (Table 1). Furthermore, when assessing the exposure to clinically relevant risk factors such as tobacco and alcohol consumption, again we noted that the groups were inherently well matched. Tobacco consumption (current or past) was analyzed as a categorical variable and was noted in 67.3% of patients in the benign category and 69.8% and 74.3% in the dysplasia and cancer categories respectively. Alcohol consumption was analyzed as a binary variable where use was noted in 70.8%, 74.4%, and 77.1% of patients in the benign, dysplasia, and cancer categories respectively. An exact chi-square test, however, did reveal a statistically significant association between risk classification and histologic diagnosis in our 161 patient cohort ($p<0.008$).

The presence of methylated *EDNRB* promoter is associated with oral premalignancy and malignancy

A univariate analysis was then conducted to evaluate the association between histologic diagnosis and variables including age, gender, race, tobacco consumption, and alcohol consumption. In the analysis, we also included methylation status of *EDNRB* and *KIF1A* as potential predictors of histology (Table 2). Based on the univariate modeling we observed that age was significantly associated with the diagnosis of benign, dysplasia, or cancer (OR=1.3, 95% CI=(1.1–1.6), p=0.014). Associations between histologic diagnosis and *EDNRB* and *KIF1A* methylation status were also found to be statistically significant (OR=3.6, 95% CI=(2.0–6.4), p<0.0001 and OR=2.2, 95% CI=(1.2–3.9), p=0.011 respectively). We did not observe a significant association with gender (p=0.618) or race (p=0.730) in the analysis. Although tobacco consumption (p=0.372) and alcohol consumption (p=0.435) did not suggest a significant association with histologic diagnosis in our cohort of patients, these variables have a well established role in the progression to oral cancer.

EDNRB and *KIF1A* were highly correlated (p<0.0001) so that their individual effects on the observed histology could not be clearly separated if they were included simultaneously in the model. We then chose *EDNRB* along with other variables of significance suggested by the univariate model (e.g., age) as well as those felt to play a clinically and biologically significant role in the development of oral cavity cancer (tobacco and alcohol consumption) to perform a multivariable proportional odds logistic regression analysis (Table 2). Once again, tobacco (p=0.454) and alcohol (p=0.663) consumption did not reach statistical significance. Age on multivariable analysis had an odds ratio of 1.2 (1.0–1.6) but only reached borderline significance (p=0.088). *EDNRB* methylation, however, remained significantly associated with histologic diagnosis (OR=3.1, 95% CI=(1.7–5.8), p=0.0003). These data indicate that *EDNRB* hypermethylation is an independent predictor of histologic diagnosis. Patients with *EDNRB* methylated were about 3 times as likely to have a diagnosis of premalignancy or malignancy as compared to those with *EDNRB* unmethylated, after adjusting for age, tobacco use, and alcohol consumption.

EDNRB salivary rinse methylation analysis can improve risk classification when combined with clinical risk classification

A subset of our patient cohort initially presented with a known diagnosis of cancer. To obtain insight into the performance of clinical risk classification and salivary rinse methylation, we excluded those patients who had a diagnosis of known cancer on presentation (n=30) from the cohort. The remaining patients (n=161) were classified using consensus risk classification methods based on clinical appearance of the oral lesion into were high risk (n=21) and low risk (n=140) lesions.[16]

We used the same predictors as used in our initial analysis and added risk classification designated during the initial examination to construct a univariate logistic regression model as shown in Table 3. Age, gender, race, tobacco, and alcohol consumption, and *KIF1A* methylation status were not significantly associated with diagnosis. Risk classification, described as low risk or high risk, based on clinical examination was significantly associated with histologic diagnosis (OR=2.5, 95% CI=(1.3–5.1), p=0.008). *EDNRB* methylation status on univariate modeling was significant (OR=2.1, 95% CI=(1.0–4.4), p=0.046). We expanded the analysis to determine if there was any correlation between EDNRB and KIF1A. A Spearman correlation coefficient was calculated as R=0.39 (p<0.0001) and a scatter plot was constructed to better visualize if there appeared to be a correlation between the two markers (Figure 1). The correlation between EDNRB and KIF1A was further evaluated by calculating the Phi Coefficient to measure the degree of correlation between two categorical variables which was 0.225. Although these results may be limited because of the use of EDNRB and

KIF!a as binary variables (methylated vs. unmethylated) and the sample size our findings suggested only a small relationship between EDNRB and KIF1A.

We then proceeded to construct a multivariable model where we analyzed risk classification, age, gender, race, tobacco, ethanol, and *EDNRB* methylation status (Table 4). Once again, risk classification remained independently associated with a histologic diagnosis (OR=2.6, 95% CI=(1.3–5.2), p=0.008). And similarly, *EDNRB* methylation demonstrated statistical significance in being independently associated with a diagnosis of dysplasia or cancer (OR=2.1, 95% CI=(1.0–4.6), p=0.047).

Through logistic regression modeling and ROC analyses, we then calculated the sensitivities and specificities of risk classification strategy and *EDNRB* methylation status (Table 5). Quantitative *EDNRB* was also considered to assess whether a cutoff other than zero may result in better predictive accuracy. The area under the curve (AUC) was also calculated with a 95% confidence interval. Risk classification as a sole predictor of histology outcome had a sensitivity of 71% (95% CI=56–83%), and a specificity of 58% (95% CI=48–67%). The AUC for risk classification was 0.65 (95% CI=0.56-.075). *EDNRB* methylation in salivary rinse samples as a predictor of histologic diagnosis had a sensitivity of 65% (95% CI= 49–78%) specificity of 51% (95% CI=42–61%) and AUC of 0.61 (95% CI=0.51–0.71) when treated as a binary value. As described in Table 5, the ROC curves with adjustment for other covariates are shown in Figure 2 demonstrating the AUC for EDNRB alone (0.61), risk classification alone (0.65), and the combination of risk classification and EDNRB (0.68).

We also included the positive (PPV) and negative (NPV) predictive values in our analysis. Individuals with a positive test had a 41% (95% CI=31–53%) chance of having high grade dysplasia/cancer based solely on risk classification. The PPV increased to 53% (95% CI=29–76%) with the combination of risk classification and EDNRB when optimizing for specificity. We saw a similar increase in the NPV with the combination of risk classification and EDNRB from 77% (95% CI=66–86%) to 83% (95% CI=72–91%) when optimizing for sensitivity.

We then performed logistic regression analysis and analyzed the variables by combining risk classification and *EDNRB* methylation to determine if there was any improvement in the predictive capability. Optimal cutoffs to maximize either sensitivity or specificity were obtained based on the predicted probability of high grade dysplasia/cancer from the multivariable logistic regression model. Using the combination of risk classification and *EDNRB* at the selected cutoff to maximize specificity resulted in a specificity of 92% (95% CI=85–96%) and sensitivity of 21% (95% CI=10–35%) with the AUC of 0.68 (95% CI=0.58–0.77). At a cutoff threshold to maximize sensitivity, addition of EDNRB methylation improved sensitivity to 75% (95% CI=60–86%) but decreased specificity from to 50% (95% CI=41–60%). In practical terms, the application of *EDNRB* salivary rinse methylation to define a high risk category of patients within a cohort of 161 patients without prior biopsy would have changed the clinical low risk assessment of 5 patients with mild dysplasia and 3 patients with severe dysplasia to high risk based on methylation analysis of salivary rinses.

DISCUSSION

Aberrant promoter hypermethylation has been recently proposed as a means for detection of HNSCC in salivary rinses. [3–7] We have studied a large cohort of prospectively collected salivary rinses obtained from patients with benign, dysplastic, and cancer diagnoses to determine the ability of Q-MSP to detect *EDNRB* or *KIF1A* promoter hypermethylation in high-risk patients. Due to the sensitivity of the Q-MSP technique used to detect the presence of *EDNRB* gene or *KIF1A* gene methylation, this enabled us to accurately correlate the risk classification strategy to the methylation status of the samples. This is the first report

demonstrating use of molecular markers in salivary rinse samples for detection of premalignant oral disease.

Our group previously published the utility of evaluating promoter region methylation status of various genes as a tool for detection of HNSCC.[12] *EDNRB* hypermethylation has been studied extensively in prostate cancers with the potential of diagnostic as well as prognostic value.[17–23] In addition, *EDNRB* methylation status has been studied in a variety of other cancers including lung cancer[24], bladder cancer[25, 26], hepatocellular carcinoma[27], nasopharyngeal carcinoma[28] and others[29, 30]. More recently, our lab demonstrated the presence of *KIF1A* promoter hypermethylation in breast cancer.[31] We have also been successful in discovering that *EDNRB* and *KIF1A* are preferentially methylated in salivary rinse samples of HNSCC (see supporting manuscript 1 available as online submission)[15] and aberrant methylation of these genes is also highly prevalent in a cohort of Indian oral squamous cell carcinoma (see supporting manuscript 2 available as online submission). Based on these prior data and data reported in various other cancers including HNSCC we selected *EDNRB* and *KIFIA* as our primary genes of interest for our study. This study evaluates promoter hypermethylation of the *EDNRB* gene and *KIF1A* gene in salivary rinse samples from patients with benign, dysplastic, and malignant lesions in combination with a risk classification strategy. Furthermore, it demonstrates the effectiveness of quantitative measurement of promoter hypermethylation in a significant sized cohort of oral cavity salivary rinse samples as a potential tool for assessing risk of malignancy, and detecting dysplastic or cancer cells.

We observed that the presence of *EDNRB* promoter methylation in salivary rinses was associated with the presence of dysplasia or invasive cancer, and that this was independent of clinical covariates including age and exposure history. This confirms that epigenetic alterations specific to dysplasia or invasive cancer can be detected in salivary rinses in the context of a dental clinic designed to assess patients at risk for oral cancer.

In our analysis we found that risk classification by a specialist resulted in a 71% sensitivity in screening indiviuals with oral cavity lesions for dysplasia or cancer. This underscores the significance of clinical examination and risk classification as a gold standard of initial screening performed by highly trained individuals such as otolaryngologists/dentists. In addition, the combination of *EDNRB* to risk classification when using a selected cutoff threshold from the logistic regression analysis allowed increased sensitivity of 75% with moderate decrease in specificity from 58% to 50%. In our analysis, we did observe considerable variablility in the sensitivity and specificity based on selected cutoff values. Ideally, we would like to observe an increase in the sensitivity without a substantial drop in the specificity with the addition of EDNRB as a predictive variable. However, we noted that even in clinical situations where a skilled professional has designated a risk classification based on clinical examination, the addition of *EDNRB* methylation status may change risk assessment of a lesion, prompting biopsy of a dysplastic or malignant oral lesion that would have otherwise not met clinical criteria for biopsy. Our examined cohort has several demographic and exposure characteristics associated with oral cancer, including tobacco and ethanol exposure and advanced age. Therefore, *EDNRB* promoter region methylation in salivary rinses status may be useful as a risk assessment tool in patients evaluated for potential oral malignancy based on exposure history and age, but presenting with a clinically low risk lesion.

The fundamental necessity of early detection in oral cancer can be confounded my numerous barriers. This can be to due the lack of a trained dentist/head and neck surgeon/otolaryngologist in the community as well as a basic lack of education/awareness in the public and health professionals.[32, 33] In addition, lack of access to health care can also prevent patients from seeking care to facilitate earlier detection.[34] The ideal test for oral premalignancy and cancer would be available for administration to a high risk population, and administered by health

care workers without specialized training, yet still provide predictive outcome results. Q-MSP provides a cost-effective easy to carry-out method that allows high-throughput and rapid analysis. This would indicate the potential use of this technique as a means for early detection of dysplastic oral cavity lesions and reinforces the potential usefulness of obtaining salivary rinses as a screening and surveillance strategy.

In a clinical setting, highly trained professionals examine patients with oral cavity lesions through conventional techniques of physical examination. Other established adjuncts include oral cytology, toluidine blue, and light-based detection systems.[35] Based on history, clinical/environmental risk factors, and oral examination parameters, the clinician sets a threshold whereby those patients that are deemed to be at a higher risk of having oral cancers undergo the gold standard scalpel biopsy. This methodology often times results in a population of patients that are categorized as low risk patients clinically, however may in fact harbor dysplastic lesions.[36] Our study unveils the presence of false negatives with using a clinically based risk classification system alone by identifying patients who had a low risk clinical lesion but had a histologic diagnosis of dysplasia. We established that the increase in sensitivity by combining both risk classification and *EDNRB* methylation status resulted from recognizing those clinical low risk patients that in fact had dysplastic lesions. Due to the lack of long term clinical outcomes for our patient cohort at this time, the true clinical implications of identifying more dysplastic lesions than would be discernable by a clinical risk stratification strategy alone needs to be interpreted with caution. Although it is not apparent which patients with dysplastic lesions may proceed to developing cancer, we acknowledge that these patients do most certainly warrant a more vigilant follow up.

The use of molecular markers in salivary rinses for the detection of cancer or those harboring occult cancers has been explored with the intent to improve screening accuracy and cost effectiveness. Salivary rinse samples potentially carry whole cells, DNA, RNA and proteins which allows for the capability of detecting alterations leading to cancer. Increasingly, saliva has been used to diagnose infectious diseases, hereditary disorders, autoimmune diseases, and endocrine disorders. Rosas *et al.* published the first study to demonstrate detection of aberrant promoter hypermethylation in saliva from HNSCC patients.[6] Carvalho *et al.* identified differential hypermethylation patterns in salivary rinses and serum of patients with HNSCC in a panel of eight genes by Q-MSP.[12] Similarly, Lallemant *et al.* analyzed the expression levels of nine genes in HNSCC and control salivary rinse samples by rT-qPCR.[4] Zhao *et al.* explored the feasibility of DNA PCR to screen for HPV in salivary rinse samples of head and neck cancer patients.[3] The novelty of this paper, however, is its prospective nature and the inclusion of salivary rinse samples from patients with premalignant lesions in the oral cavity. Further investigations of the functionality of *EDNRB* and downstream pathways may yield additional insight to its role in oral cavity lesions.

Future studies using CpG island microarray technologies may be useful in creating helpful panels of genes with increased sensitivities and retained specificities. Future studies to investigate the progression from premalignant changes to malignant transformation and the timing of the aberrant hypermethylation will also be of great value. Nonetheless, the use of salivary rinse molecular analysis may offer a feasible, rapid and cost-effective tool for stratification of high-risk patients and early detection of premalignant lesions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

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

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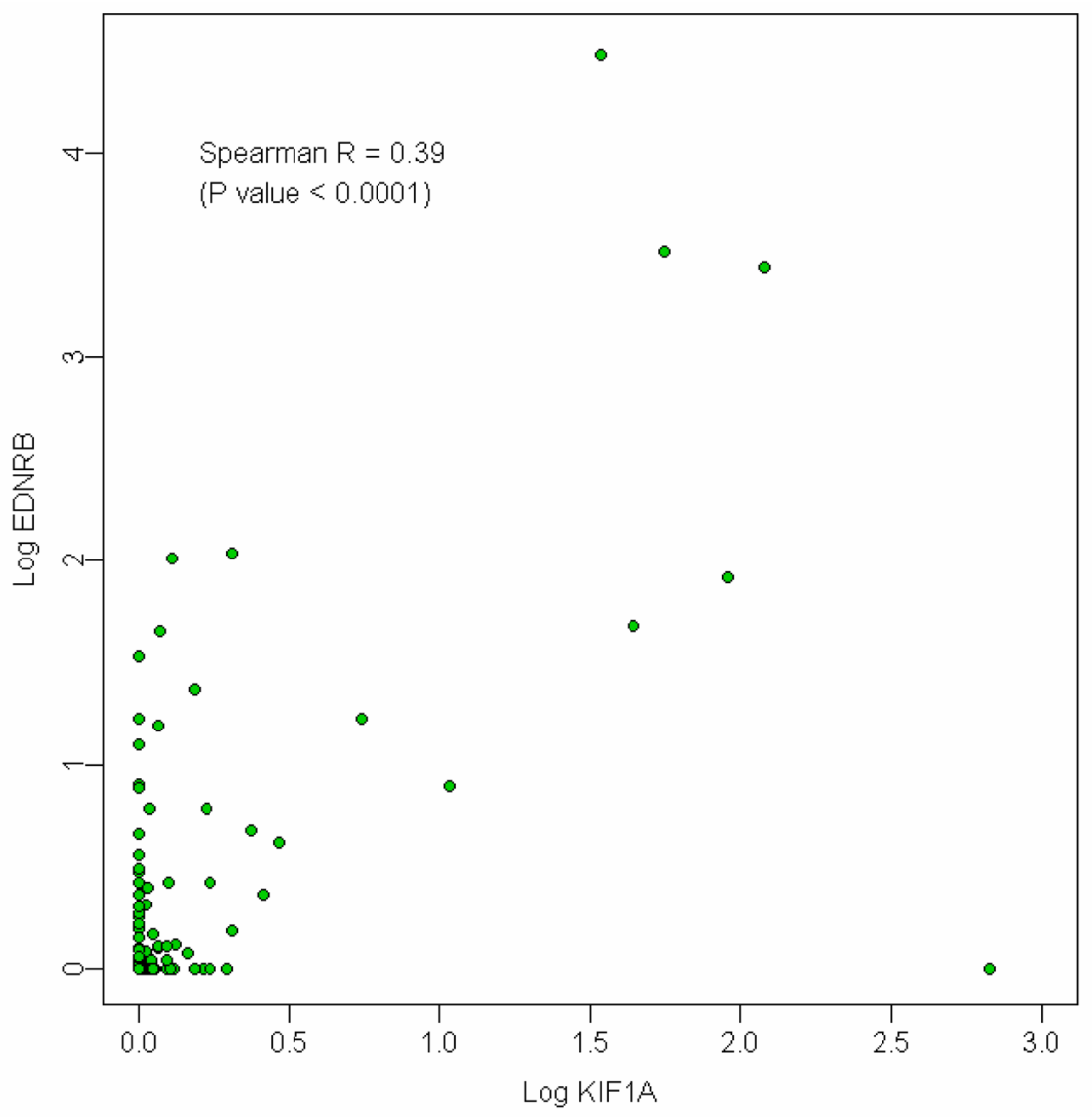

The above scatter plot provides a visual observation of correlation between numerical EDNRB and KIF1A (log transformed).

Figure 1.
Correlation between EDNRB and KIF1A

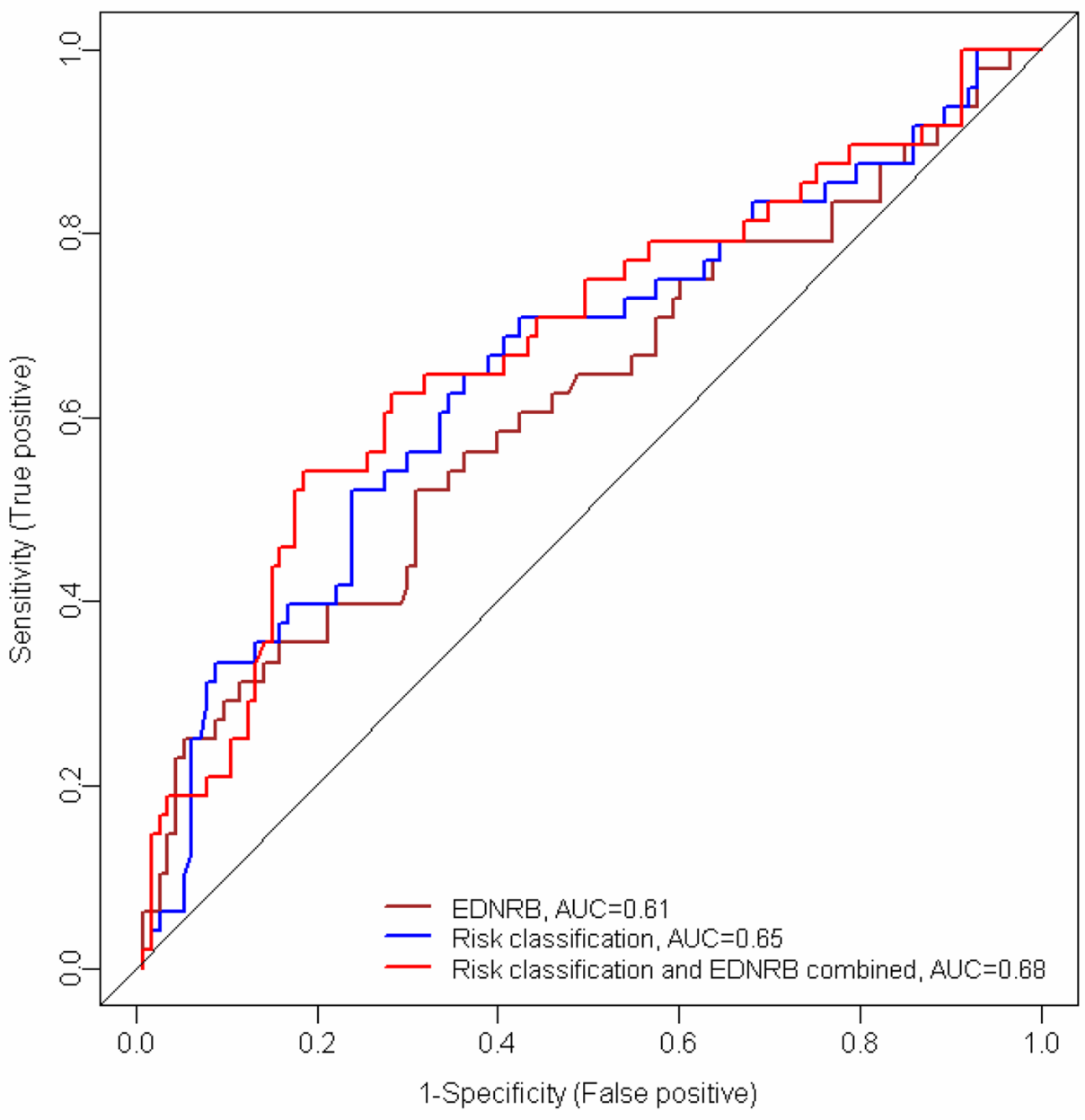

Figure 2.
ROC curves with adjustment for other covariates as described in Table 5

Table 1

Univariate analysis of association between predictors and histology (n=191)

Variable	Benign (n=113)	Premalignant (n=43)	Malignant (n=35)	Total
Age (years)				
Mean (±SD)	52 (± 13)	56 (± 14)	58 (± 14)	54 (± 13)
Median (range)	52 (18 – 78)	57 (26 – 82)	56 (24 – 90)	54 (18 – 90)
Gender, n [%]				
Female	36 (31.9)	14 (32.6)	9 (25.7)	59 (30.9)
Male	77 (68.1)	29 (67.4)	26 (74.3)	132 (69.1)
Race, n [%]				
African-American	28 (24.8)	9 (20.9)	7 (20.0)	44 (23.0)
Caucasian	77 (68.1)	32 (74.4)	24 (68.6)	133 (69.6)
Other	8 (7.1)	2 (4.7)	4 (11.4)	14 (7.3)
Tobacco, n [%]				
Never user	37 (32.7)	13 (30.2)	9 (25.7)	59 (30.9)
Former user	20 (17.7)	7 (16.3)	11 (31.4)	38 (19.9)
Current user	56 (49.6)	23 (53.5)	15 (42.9)	94 (49.2)
Ethanol, n [%]				
Never used	33 (29.2)	11 (25.6)	8 (22.9)	52 (27.2)
Used	80 (70.8)	32 (74.4)	27 (77.1)	139 (72.8)
Risk classification, n [%]				
Low risk	75 (66.4)	21 (48.8)	0 (0)	96 (50.3)
High risk	38 (33.6)	21 (48.8)	6 (17.1)	65 (34.0)
Cancer	0 (0)	1 (2.3)	29 (82.9)	30 (15.7)
EDNRB, n [%]				
Unmethylated	88 (77.9)	26 (60.5)	14 (40.0)	128 (67.0)
Methylated	25 (22.1)	17 (39.5)	21 (60.0)	63 (33.0)
KIF1A, n [%]				
Unmethylated	84 (74.3)	31 (72.1)	17 (48.6)	132 (69.1)
Methylated	28 (24.8)	12 (27.9)	18 (51.4)	58 (30.4)
Missing	1 (0.9)	0 (0)	0 (0)	1 (0.5)

Table 2

Analyses of association between predictors and histology (n=191) -EDNRB

Variable	Univariate analysis		Multivariable analysis	
	OR[1] (95% CI)	P[1]	Adjusted OR[2] (95% CI)	Adjusted P[2]
Age (years)	1.3[3] (1.1 – 1.6)	0.014	1.2[3] (1.0 – 1.6)	0.088
Gender				
Female	Ref		Ref	
Male	1.2 (0.6 – 2.1)	0.618	0.9 (0.5 – 1.9)	0.896
Race				
African-American	Ref		Ref	
Caucasian	1.2 (0.6 – 2.5)	0.730	1.1 (0.6 – 2.4)	0.745
Other	1.5 (0.5 – 4.9)		1.6 (0.5 – 5.5)	
Tobacco				
Never user	Ref		Ref	
Former user	1.7 (0.8 – 3.8)	0.372	1.7 (0.7 – 3.9)	0.454
Current user	1.1 (0.6 – 2.2)		1.4 (0.7 – 3.0)	
Ethanol				
Never used	Ref		Ref	
Used	1.3 (0.7 – 2.4)	0.435	1.2 (0.6 – 2.3)	0.663
EDNRB				
Unmethylated	Ref		Ref	
Methylated	3.6 (2.0 – 6.4)	< .0001	3.1 (1.7 – 5.8)	0.0003

[1] Univariate proportional odds model;

[2] Multivariate proportional odds model;

[3] Unit of 10 years

Table 3

Univariate analysis of association between predictors and histology – excluding patients with known cancer at presentation (n=161)

Variable	Benign (n=113)	Dysplasia/cancer (n=48)	Odds ratio[1] (95% CI)	P[1]
Age (years)				
Mean (± SD)	52 (± 13)	56 (± 15)	1.2[2] (0.9 – 1.6)	0.110
Median (range)	52 (18 – 78)	57 (24 – 86)		
Gender, n [%]				
Female	36 (31.9)	15 (31.3)	Ref	
Male	77 (68.1)	33 (68.7)	1.0 (0.5 – 2.1)	0.940
Race, n [%]				
African-American	28 (24.8)	10 (20.8)	Ref	
White	77 (68.1)	36 (75.0)	1.3 (0.6 – 3.0)	0.639
Other	8 (7.1)	2 (4.2)	0.7 (0.1 – 3.9)	
Tobacco, n [%]				
Never user	37 (32.7)	14 (29.2)	Ref	
Former user	20 (17.7)	10 (20.8)	1.3 (0.5 – 3.5)	0.855
Current user	56 (49.6)	24 (50.0)	1.1 (0.5 – 2.5)	
Ethanol, n [%]				
Neverused	33 (29.2)	12 (25.0)	Ref	
Used	80 (70.8)	36 (75.0)	1.2 (0.6 – 2.7)	0.587
Risk classification, n [%]				
Low risk	75 (66.4)	21 (43.8)	Ref	
High risk	38 (33.6)	27 (56.2)	2.5 (1.3 – 5.1)	0.008
EDNRB, n [%]				
Unmethylated	88 (77.9)	30 (62.5)	Ref	
Methylated	25 (22.1)	18 (37.5)	2.1 (1.0 – 4.4)	0.046
KIF1A, n [%]				
Unmethylated	84 (74.3)	35 (72.9)	Ref	
Methylated	28 (24.8)	13 (27.1)	1.1 (0.5 – 2.4)	0.782
Missing[3]	1 (0.9)	0 (0)		

[1] Univariate logistic regression model;

[2] Unit of 10 years;

[3] Excluded from the analysis of association

Table 4

Risk factors for histologic diagnosis by multivariable analysis(n=161)

Variable	Adjusted OR[1] (95% CI)	Adjusted P[1]
Age (years)	1.1[2] (0.8 – 1.5)	0.455
Gender		
Male vs. female	0.9 (0.4 – 2.0)	0.763
Race		
Caucasian vs. African-American	1.3 (0.5 – 3.2)	0.547
Othervs. African-American	0.7 (0.1 – 4.0)	0.659
Tobacco		
Former uservs. never user	1.5 (0.5 – 4.4)	0.457
Currentuservs. never user	1.6 (0.7 – 3.8)	0.296
Ethanol		
Used vs. never used	1.3 (0.6 – 3.0)	0.561
Risk classification		
High risk vs. low risk	2.6(1.3 – 5.2)	0.008
EDNRB		
Methylated vs. unmethylated	2.1 (1.0 – 4.6)	0.047

[1]Multivariate logistic regression model;

[2]Unit of 10 years

Table 5

Predictive accuracy of risk classification and EDNRB and combination after adjusting for other predictors[1] associated with head and neck cancer (n=161)

Predictor	Cutoff[2]	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV[3] (%, 95% CI)	NPV[4] (%, 95% CI)	AUC (95% CI)
Risk classification	0.267590	71(56 – 83)	58(48 – 67)	41 (31 – 53)	77 (66 – 86)	0.65(0.56 – 0.75)
EDNRB	0.274459	65(49 – 78)	51(42 – 61)	36 (26 – 47)	82 (72 – 90)	0.61 (0.51 – 0.71)
Risk and EDNRB	0.246792[1]	75(60 – 86)	50(41 – 60)	39 (29 – 50)	83 (72 – 91)	0.68(0.58 – 0.77)
combined	0.450188[1]	21 (10 – 35)	92(85 – 96)	53 (29 – 76)	73 (65 – 80)	

[1] Other predictors include age, sex, race, tobacco, and ethanol use;

[2] Based on predicted probability of high grade dysplasia/cancerusing multivariable logistic regression model;

[3] Positive predictive value, depending on the prevalence of the disease (high grade dysplasia/cancer) which was 13% for this study population.

[4] Negative predictive value, depending on the prevalence of the disease (high grade dysplasia/cancer) which was 13% for this study population.

Note: The cutoffs are not EDNRB methylation values but a predicted probability from the logistic regression model that simultaneously includes risk classification and EDNRB.

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