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Detection of TIMP3 promoter hypermethylation in salivary rinse as an independent predictor of local recurrence-free survival in head and neck cancer

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Abstract

Purpose—To validate a panel of methylation-based salivary rinse biomarkers (P16, CCNA1, DCC, TIMP3, MGMT, DAPK, and MINT31) previously shown to be independently associated with poor overall survival and local recurrence in a larger, separate cohort of patients with head and neck squamous cell carcinoma (HNSCC).

Experimental Design—One hundred ninety-seven patients were included. All pre-treatment saliva DNA samples were evaluated for the methylation status of the gene promoters by quantitative methylation-specific PCR. The main outcome measures were overall survival, local recurrence-free survival and disease-free survival.

Results—In univariate analyses, the detection of hypermethylation of CCNA1, MGMT, and MINT31 was significantly associated with poor overall survival; the detection of hypermethylation of TIMP3 was significantly associated with local recurrence-free survival; and the detection of hypermethylation of MINT31 was significantly associated with poor disease-free survival. In multivariate analyses, detection of hypermethylation at any single marker was not predictive of overall survival in patients with HNSCC; detection of hypermethylation of TIMP3 in salivary rinse had an independent, significant association with local recurrence-free survival (Hazard Ratio, 2.51, 95% CI, 1.10 to 5.68); and none of the studied markers was significantly associated with disease-free survival.

Conclusion—The detection of promoter hypermethylation of the seven genes in salivary rinse as an independent prognostic indicator of overall survival in patients with HNSCC was not validated. Detection of promoter hypermethylation of TIMP3 in pretreatment salivary rinse is independently associated with local recurrence-free survival in patients with HNSCC and may be a valuable salivary rinse biomarker for HNSCC recurrence.

INTRODUCTION

Over 50,000 new cases of head and neck squamous cell carcinoma (HNSCC) are diagnosed in the United States yearly, with a mortality rate of 12, 000 annually. As with lung cancer, this malignancy is also predominantly related to smoking with alcohol as a co-carcinogen, although infection with the human papillomavirus (HPV) has also been associated with the majority of oropharynx cancers (1, 2). Despite significant progress in therapeutic interventions, including surgery, radiotherapy, and chemotherapy, the 5-year survival rate for patients with HNSCC has shown only modest improvement in the past decades (3). Treatment of HNSCC involves several challenges, including local control of primary tumor. Primary tumor recurrence is a significant contributor to disease morbidity, but even successful treatment of primary occurrences can result in significant morbidity, including dysphagia, dysarthria with surgical salvage requiring laryngectomy as well as other procedures (4). Despite combined modality therapy, local recurrence still occurs in at least 15% of cases, with higher rates in many series depending on site and stage (5-7). Intuitively, using molecular biomarkers that could predict the likelihood of survival or recurrence may direct the extent of therapy with better outcomes.

Epigenetic gene silencing is a molecular mechanism of silencing a gene by methylating its promoter region and plays a vital role(s) in the development of several types of cancer, including HNSCC (8-10). Aberrant promoter methylation may affect genes involved in cell cycle control (P16, Rb, and P14) (11-13), DNA repair (MGMT and hMLH1) (14, 15), cell adhesion (H-cadherin and CDH-1) (16, 17), signal transduction (RASSF1A) (18), apoptosis (DAPK and TMS1) (19) and cell differentiation (RARβ2) (20). Promoter hypermethylation in tissue samples can be detected using quantitative methylation-specific PCR. This real-time PCR methodology allows a more objective, robust, and rapid assessment of promoter methylation status. The ability to quantify methylation provides the potential for determination of a threshold level to improve sensitivity and specificity in detection of tumor specific signal (21-23). Recent publications have shown the detection of promoter hypermethylation in various bodily fluids including saliva (24-26). The detection of DNA methylation in bodily fluids opens the potential to develop of biomarkers predictive of local recurrence and poor survival.

We have previously published results of salivary rinse screening using promoter hypermethylation based-markers in patients with previously diagnosed HNSCC. We developed a panel of promoter hypermethylation markers, including DAPK, DCC, MINT-31, TIMP3, P16, MGMT, and CCNA1 for detection of HNSCC by evaluation of salivary rinse from these patients (27). Further, in a pilot cohort of 61 HNSCC patients, we reported the potential of detection of promoter hypermethylation of this panel in pretreatment salivary rinses as a biomarker for HNSCC surveillance (28). In the current study, we intended to validate the biomarker panel status of salivary rinses from a larger, separate, prospectively collected cohort of patients with HNSCC. The independent association of biomarker status with overall survival, disease free survival and local recurrence in this cohort were determined.

MATERIALS AND METHODS

Subjects

Between August 1999 and January 2010, the salivary rinse samples were prospectively collected from patients (n=197) presenting with previously untreated squamous cell carcinoma from the oral cavity, larynx, or pharynx, at the Department of Otolaryngology-Head and Neck Surgery, Johns Hopkins Medical Institutions (Baltimore, MD). Institutional approval from our Institutional Review Board was obtained for the conduct of the studies.

According to our salivary rinse collection protocol, a written informed consent was obtained from each subject. No selection criteria were applied on patients. To obtain clinical information, we reviewed medical records of patients with pathologically confirmed HNSCC. Enrollment included collection of demographic information and risk factor history. Smoking was defined as use of tobacco, chewable or smoked, for at least 1 year continuously. Patient follow-up information was obtained through review of hospital and physician records, direct patient contact, the tumor registry, and the social security death index. The median follow up time of these patients is 29.1 months (range 0.2-115.0 months) after the collection of the salivary rinse samples.

Collection of salivary rinse samples

Salivary rinses were obtained before tumor treatment from all subjects as previously described (27). In brief, the tissue collected using this technique includes exfoliated epithelial cells from the upper aerodigestive tract, and an exfoliating brush is used to harvest cells from deep epithelial layers in the oral cavity and oropharynx. The tumor site is intentionally avoided during brushing. This technique allows for a broad sampling of epithelial cells from multiple sites in the upper aerodigestive tract. Salivary rinses were obtained by using rinsing and gargling with 20 mL of normal saline solution. Cellular material from the brushing was released into the saline rinse and centrifuged to obtain a cell pellet after supernatant was discarded. Pellets were then immediately frozen and stored at -80°C .

DNA extraction

DNA obtained from salivary rinse samples was extracted by the tissue bank by digestion with 50 $\mu\text{g/mL}$ 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 was subjected to bisulfite treatment, using the EpiTect Bisulfite kit from Qiagen according to the manufacturer's conditions, <http://www.Qiagen.com>. Bisulfite-treated DNA was eluted in 30 μL of elution buffer and stored at -80°C (29, 30).

Quantitative methylation-specific PCR

The bisulfite-treated DNA was used as a template for fluorescence-based real-time Q-MSP as described previously (31). The P16, CCNA1, DCC, TIMP3, MGMT, DAPK, MINT31 and ACTB genes had been previously detected on a prior screen of salivary rinses in HNSCC patients. All of these markers were found to be present in negligible amount in the saliva of healthy control subjects (27, 28). We had previously optimized the primer and probe sequences for Q-MSP, and their sequences are available on previous publication (27). The ratios between the values of the gene of interest and the reference gene ACTB were 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 ACTB gene $\times$ 100). Fluorogenic PCRs were carried out in a reaction volume of 10 μL of 200 nmol/L of each primer; 100 nmol/L of probe; 0.375 units of platinum Taq Polymerase (Invitrogen); 100 $\mu\text{mol/L}$ of ROX Reference Dye (Invitrogen); 8.4 mmol/L ammonium sulfate; 33.5 mmol/L Trizma (Sigma); 3.35 mmol/L magnesium chloride; 5 mmol/L mercaptoethanol; and 0.05% DMSO. Each real-time Q-MSP reaction consisted of 1.5 μL 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 2 minutes followed by 50 cycles of 95°C for 15 seconds and 60°C for 1

minute. 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 done by collecting leukocytes from a healthy individual and subjecting the cells to methylation in vitro with excess SssI methyltransferase (New England Biolabs) to generate completely methylated DNA. The DNA was then bisulfite treated as described above. Serial dilutions of the 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 done on the samples. These samples were used as a negative control for the reactions. Leukocyte DNA from a healthy individual was also methylated in vitro with excess SssI methyltransferase (New England Biolabs) to generate completely methylated DNA, and serial dilutions (45-0.0045 ng) of this DNA were used to construct a calibration curve for each plate (32). There were also several control wells in each plate that contained only the reaction mix and water to ensure that there was no contamination. The results of Q-MSP were analyzed considering the quantity of methylation normalized by ACTB as well as the quantity of methylation as a binary event, in which any quantity of methylation in a sample would be considered positive for methylation.

Target gene selection

Genes selected for this study came from a study previously done in our lab to develop a panel for HNSCC detection and surveillance in body fluids (27, 28). These genes included P16, CCNA1, DCC, TIMP3, MGMT, DAPK, and MINT31. In our former study, we reported the aberrant promoter hypermethylation of P16, MGMT, DAPK (26), TIMP3 (33), CCNA1 (34), DCC (35) in HNSCC tumor tissues. We also reported aberrant promoter hypermethylation of CCNA1 in O22 and O28 HNSCC cell lines (34) and DCC in O12 HNSCC cell lines (35). Ogi K. et al. recently reported the aberrant promoter hypermethylation of MINT31 in HNSCC tumor tissues (36).

HPV analysis

The HPV status was determined as described previously (28, 37). In brief, specific primers and probes have been designed to amplify the E6, E7 regions of HPV16. Their sequences are available in a previous publication (28). All the samples were run in duplicate. Primers and probes to a house-keeping gene (β -actin) were run in duplicate and parallel to normalize input DNA. Samples in which two results were not concordant were repeated twice in duplicate and were usually due to failed PCR in one of the initial reactions. Each reaction was run 50 cycles. By using serial dilutions, standard curves were developed for the HPV 16 viral copy number using CaSki (American Type Culture Collection, Manassas, VA) cell line genomic DNA, known to have 600 copies/genome (6.6 pg of DNA/genome). Standard curves were developed for HPV16 E6 and E7, using serial dilutions of DNA extracted from CaSki cells with 50,000 pg, 5,000 pg, 500 pg, 50 pg and 5 pg of DNA. Standard curves were developed as well for the β -actin housekeeping gene (2 copies / genome), using the same serial dilutions of the CaSki genomic DNA. This additional step allowed for relative quantification of the input DNA level and final quantity as the number of viral copies/genome/cell. HPV copy number > 0.1 copy/cell for tumor samples were regarded as positive. For saliva samples, any amplified sample with HPV E6 or E7 amplification with at control β -actin amplification of 10 ng was regarded as positive.

Statistical analysis

Hypermethylation of each gene was treated as a binary variable (methylation versus no methylation) by dichotomizing the methylation at zero. Factors tested for prognostic value includes the age, sex, race, smoking status, alcohol use, HPV status, primary tumor site, pathological tumor stage, pathological nodal stage, clinical TNM stage, margin status, and the presence of promoter methylation of P16, CCNA1, DCC, TIMP3, MGMT, DAPK, or

MINT31. For each patient characteristic and/or clinical parameter, patients with missing information were considered as a separate category and included in the analysis as well. In each case, we verified whether the category “missing information” was associated with a significant prognostic value.

The primary end points of the study were overall survival, disease free survival, and local recurrence free survival. Overall survival was defined as the time elapsed from the date of completion of therapy to the date of death from any cause or the date of last follow-up. Disease-free survival was defined as the time elapsed from the date of completion of therapy to the date of the first adverse event (i.e, persistent disease, disease recurrence or death without recurrence). Local recurrence free survival was defined as the time elapsed from the date of completion of therapy to the date of local recurrence. Death without local recurrence is considered as a competing risk for local recurrence.

Proportional-hazards models were used to assess the univariate prognostic significance of clinical variables and each individual methylation marker on overall survival and disease free survival. Computing risk regression model using the Fine and Gray method (38) was applied for the local recurrence free survival outcome. The hazard ratios were calculated relative to a reference group and presented with their corresponding 95% CIs. Multivariable analysis was performed to evaluate the effect of the methylation markers on risk of failure, adjusting for each patient and tumor characteristic prognostic factors. Methylation indicator of each of the seven genes as well as additional prognostic factors whose P value were lower than 0.05 was included in the multivariate Cox regression model.

The analysis was performed using statistical package SAS. Competing risk package cmprsk in R (<http://cran.rproject.org/doc/cmprsk.pdf>) was used for the computation of computing risk regression for the local recurrence free survival endpoint. Corrected group prognostic curves of cumulative incidence function for local recurrence survival was generated with adjustment for covariates (39). In the sensitivity analyses, we either included death without recurrence as an event, or censored those patients who died. All reported P values are two-sided, P value less than 0.05 were considered statistically significant.

RESULTS

1. Characteristics of the Patients

The study population consisted of 197 patients with a historically confirmed diagnosis of HNSCC from August 1999 through January 2010. The characteristics of the study population (n=197) largely reflect the demographics of head and neck cancer patients in the United States (Table I). The HNSCC patients were mainly males (76.1%, 150/197) and Caucasians (86.3%, 170/197), with ages range from 25 to 87 years (median, 58 years). Smoking and alcohol consumption was found in 67.0% (132/197) and 67.7% (133/197) respectively. With regard to HPV status, the study population consisted of 44.7% (88/197) HPV-positive patients. The primary tumor was located in the oral cavity (n=53, 26.9%), oropharynx (n=102, 51.8%), hypopharynx (n=6, 3.0%), or larynx (n=27, 13.7%). Sixty-six percent of the patients (130 of 197) presented with locally advanced stage IV disease. Nodal status was N1 or N2 in 68.5% (135 of 197) patients. Positive margins were noted in 41.8% (64 of 153) of surgically treated patients. The distribution of the patients according to primary therapy were surgery (n=153, 77.7%), radiation therapy (n=6, 3.0%), chemoradiotherapy (n=29, 14.7%) and other/combination (n=9, 6.6%). The median follow up time of these patients is 29.1 months (range 0.2 - 115.0 months) after the collection of the salivary rinse samples, with 63 (32.0%) individuals having more than 4 years follow-up. As of December 2010, a total of 60 patients have died. The cause death was head and neck cancer in 34 patients, other causes in 26. At the end of the follow-up period, there were six

patients alive with disease. During this period, 36 (18.3%) recurrences were detected, including 12 of local recurrence, 6 of locoregional recurrence, 1 of local and distant recurrence, 2 of locoregional and distant recurrence.

2. Promoter Hypermethylation of P16, CCNA1, DCC, TIMP3, MGMT, DAPK, and MINT31

We first tested the promoter methylation status of seven genes (P16, CCNA1, DCC, TIMP3, MGMT, DAPK, and MINT31) in the salivary rinses from the above 197 HNSCC patients using QMSP. Of the 197 salivary rinse samples, 12 were eliminated for the QMSP analysis (8 due to inadequate salivary rinse DNA and 4 due to poor quality DNA (β -actin could not be amplified)). The methylation status was determined in the remaining 185 salivary rinse samples from patients with HNSCC. The patterns of promoter methylation of these seven genes are shown in Figure 1. Among the salivary rinse samples detected, promoter methylation was found in 7.6% (14/185) at P16, 15.2% (28/185) at CCNA1, 22.7% (42/185) at DCC, 20.5% (38/185) at TIMP3, 22.1% (41/185) at MGMT, 9.2% (17/185) at DAPK, and 5.4% (10/185) at MINT31. Approximately 47% (87/185) salivary rinses had promoter hypermethylation of at least one gene of these seven genes.

3. Overall Survival

Univariate analysis of clinical and pathologic characteristics indicated that the covariates of age (Hazard Ratio, 1.06; 95% CI, 1.04 to 1.09), HPV status (Hazard Ratio, 0.30, 95% CI, 0.16 to 0.57), primary tumor site (Oropharynx vs Other: Hazard Ratio, 0.39, 95% CI, 0.22 to 0.67), pathologic tumor stage (T3/T4, vs T1/T2: Hazard Ratio, 4.07; 95% CI, 2.39 to 6.92), and margin status (Cancer vs Other, Hazard Ratio, 1.83, 95% CI, 1.08 to 3.10) were significantly associated with overall survival whereas no significant prognostic values were found with the other factors (Table II). The presence of promoter hypermethylation of CCNA1, MGMT, and MINT31 in salivary rinses was significantly associated with overall survival. In the patients in which the presence of promoter methylation of CCNA1, MGMT, or MINT31 was observed in salivary rinses, the hazard ratios for overall survival were 1.92 (95% CI, 0.99 to 3.72), 1.93 (95% CI, 1.10 to 3.39), 4.42 (95% CI, 1.88 to 10.41), respectively (Table II). Yet, in the multivariate analysis, with the adjustment of age, HPV status, primary tumor site, pathologic tumor stage, and margin status, the detection of promoter methylation of CCNA1, MGMT, or MINT31 did not reach statistical significance as predictors of overall survival (Table III, and Supplementary Table I).

4. Disease-Free Survival

In univariate analysis, we found that traditional markers for prognosis including age (Hazard Ratio, 1.05; 95% CI, 1.03 to 1.07), HPV status (Hazard Ratio, 0.35; 95% CI, 0.20 to 0.60), primary tumor site (Oropharynx vs Other, Hazard Ratio, 0.43; 95% CI, 0.27 to 0.70), pathological tumor stage (Hazard Ratio, 2.77; 95% CI, 1.75 to 4.39), were associated with disease-free survival. Among the seven methylation markers, the presence of promoter methylation of MINT31 was significant associated with disease free survival (Hazard Ratio, 2.86, 95% CI, 1.23 to 6.67), while the presence of promoter methylation of CCNA1 and MGMT were only marginally associated with disease free survival (CCNA1, Hazard Ratio, 1.72, 95% CI, 0.94 to 3.15; MGMT, Hazard Ratio, 1.57, 95% CI, 0.93 to 2.64) (Table II). In a Cox regression analysis, after adjusting for age, HPV, primary tumor site, pathological tumor stage, the presence of promoter methylation of MINT31 in salivary rinse samples did not achieve statistical significance as predictor of disease free survival (Table III, and Supplementary Table II).

5. Local Recurrence-Free Survival

The relation between the presence of promoter hypermethylation of these seven genes and local recurrence was then investigated to identify biomarkers that would be useful as a risk factor of local recurrence. Because in the 197 patients we studied, 24 died of other causes before local recurrence. Therefore, during our analysis we considered death before local recurrence as a competing risk, instead of treating it as an event or censoring it. For patients with the presence of TIMP3 promoter hypermethylation, the 5-year cumulative incidence of local recurrence was 23.7% compared with 11.7% for patients without the presence of TIMP3 promoter hypermethylation. In our competing risk regression model using Fine and Gray method, the presence of promoter methylation of TIMP3 was found significantly associated with local recurrence free survival (Hazard Ratio, 2.61, 95% CI, 1.16 to 5.84, Table II). Moreover, when analyzed in multivariate analysis adjusting for other prognostic factor of primary tumor site (primary tumor site was the only clinical factor showing marginally statistical significance with local recurrence in univariate analysis, Oropharynx vs Other, Hazard Ratio, 0.45, 95% CI, 0.21 to 1.01, Table II), the presence of TIMP3 promoter methylation remained an independent prognostic factor for local recurrence free survival (Hazard Ratio, 2.51, 95% CI, 1.10 to 5.68) (Table III, and Supplementary Table III). Figure 2 presented the cumulative incidence of local recurrence for patients between present or absent of TIMP3 promoter hypermethylation ($P=0.02$) with adjustment of primary tumor site. A similar result was also produced when using non-parametric method (data not shown). In addition, to confirm this result, two sensitivity analyses were undertaken with one where death without local recurrence is included as an event and the other with death treated as censored. In both sensitivity analyses, the presence of TIMP3 promoter methylation was consistently shown as an independent factor for local recurrence overall survival when adjusting for other prognostic factors (data not shown).

DISCUSSION

Body fluids such as saliva, which can be obtained by noninvasive techniques, are potential sources for development of biomarkers for detection, diagnosis and prognosis of HNSCC. Previous research demonstrated the feasibility of detection of promoter hypermethylation using salivary rinses as a means for detection of HNSCC. Recently, we studied a large sample size of both controls and HNSCC patients using multiple panels of methylated promoter regions with quantitative-MSP in salivary rinses. From the initial screening of 21 genes for salivary rinses, ultimately, seven genes, including DAPK, DCC, MINT-31, TIMP3, P16, MGMT and CCNA1, were selected as part of a panel to distinguish salivary rinses from HNSCC patients and healthy controls (27). With a pilot cohort of 61 HNSCC patients, we also found that the detection of these markers in pretreatment salivary rinse DNA is likely prognostic indicator for local recurrence and poor survival (28). In this current study, we employed a separate, prospectively collected cohort of 197 patients with HNSCC to validate the association of these seven methylation-based salivary-rinse biomarkers with survival and local recurrence.

Our data indicated that detection of promoter methylation of CCNA1, MGMT, and MINT31 in salivary rinses was significantly associated with poor overall survival of patients with HNSCC in univariate analysis. Yet, none of them are independent prognostic factors as shown in multivariate analysis. Our data also supported that detection of promoter hypermethylation of MINT31 was significantly associated with poor disease-free survival in patients with HNSCC, whereas detection of promoter hypermethylation of CCNA1 or MGMT was of borderline significance, as shown in univariate analysis. However no association was found between detection of promoter methylation of MINT31, CCNA1, or MGMT in salivary rinse with disease free survival for patients with HNSCC in multivariate analysis. It is thus unlikely that methylation of any one of the individual makers examined in

salivary rinse was for overall survival or disease-free survival observed with patients with HNSCC. No single methylation marker was associated with outcome in multivariate analysis to alone account for a difference in the clinical course of disease. Clearly, we have not eliminated the possibility that methylation of one or more other critical genes has occurred and that this process is indirectly associated methylation of our gene panel.

We were not able to fully validate the independent prognostic significance (overall survival) of our initial marker panels identified by our pilot study in this larger, separately collected cohort. With the current cohort, even when the subset of patients whose primary site is oral cavity was analyzed alone, the independent prognostic association of promoter hypermethylation of this salivary rinse marker panel with overall survival was not able to be validated (data not shown). It is difficult to speculate on potential factors affecting our results. To date, the mechanism leading to the presence of gene promoter hypermethylation in salivary rinse is not well understood. Previously, we proposed that 1) aggressive tumors may undergo increase rate of mechanical dissociation or shedding into salivary rinses. Those tumors with a higher burden of epigenetic alteration may be more frequently detected in salivary rinses, and may have a more aggressive behavior; 2) premalignant clonal patches expand well beyond primary tumor location, resulting a larger surface area of epigenetically altered cells to shed into the saliva, and also may predispose to development of recurrent tumors from adjacent premalignant cells (28). The demographic and clinical characteristics of our current cohort appeared comparable to that in the pilot study we previously published, and were broadly representative of standard clinical practice. The large sample size of this validation cohort should provide additional power to determine the significance of marker panel combinations with borderline significance in our pilot study. It is possible that these associations could have occurred by chance alone and should be interpreted with caution.

Importantly, our study demonstrated a significant association between detection of TIMP3 promoter hypermethylation in salivary rinse and local recurrence free survival. We reported that HNSCC patients with promoter hypermethylation of TIMP3 in salivary rinses have a poorer local recurrence-free survival than those without in the univariate analysis (Hazard Ratio, 2.61, 95% CI, 1.16 to 5.84). Furthermore, multivariate analysis confirmed detection of TIMP3 promoter hypermethylation in salivary rinse as an independent prognostic marker of local recurrence-free survival (Hazard Ratio, 2.51, 95% CI, 1.10 to 5.68).

TIMP3 is a tumor suppressor gene found on chromosome 12q12.3 and is part of a family of genes that inhibit matrix metalloproteinase (MMPs). TIMP3 regulates activities of MMPs and functions dependent on matrix composition including invasion, migration, differentiation, and proliferation. Promoter hypermethylation of TIMP3 has been shown to be a pathway to carcinogenesis in pancreatic, gastric, kidney, brain, colorectal, lung, breast, and several other tumor types (40-43). In HNSCC, we reported that TIMP3 promoter hypermethylation is elevated in HNSCC and is highly correlated with DAPK hypermethylation, implying a functional relationship between these genes (33). Recently, De Schutter et al. found that promoter methylation of TIMP3 predicts better outcome in patients with HNSCC treated by radiotherapy only (44). Ninomiya et al suggested that TIMP3 hypermethylation in esophageal squamous cell carcinoma had significantly poorer prognosis compared to those without (45). Of note, Righini et al. identified one case with HNSCC relapse showing positive TIMP3 promoter hypermethylation in saliva (46). To our knowledge, this is the first study demonstrating detection of TIMP3 promoter hypermethylation in pretreatment salivary rinse as a prognostic indicator of local recurrence-free survival in patients with HNSCC. Incorporation of this methylation marker into clinical practice may result in more accurate prediction of patients with previously treated HNSCC who are most likely to experience recurrence. Obviously, validation of TIMP promoter

methylation in salivary rinses in an independent, expanded cohort would be needed to validate the prognostic significance of this biomarker.

We also propose that it may not necessary to perform comparative analysis with tumor tissues to address the heterogeneity of these salivary rinse methylation markers in the current study. Given that the purpose of this study is to validate the association between a panel of methylation-based salivary rinse biomarkers and head and neck cancer surveillance based on our previously published data, we have applied the same methodology used in our former studies for marker validation (27, 28). Biologically, HNSCC often develop within preneoplastic fields of mucosal epithelium made up of genetically altered cells (2). The concept of “field cancerization” is defined as the presence of one or more mucosal areas consisting of epithelial cells that have cancer-associated genetic or epigenetic alterations. A field is preneoplastic by definition, it may have histological aberrations characteristic of dysplasia, but not necessarily. This postulates that insult from a carcinogen occurs across the entire epithelial field, giving rise to multiple, independent sites of carcinogenesis (47). The concept of “field cancerization” has being applied to the head and neck cancers that occur in oral cancer, laryngeal cancer, hypopharyngeal cancer, and partially in oropharyngeal cancer (in our validation cohort, 51.8% are oropharyngeal cancer). Notably, in our former study examining promoter hypermethylation pattern of p16, MGMT and DAPK in tumors and saliva of 30 head and neck cancer patients, we have compared their promoter hypermethylation pattern between tumors and saliva. The promoter hypermethylation of p16, MGMT and DAPK were detected consistently positive or negative in 90% (27/30), 86.7% (26/30) and 86.7% (26/30%) of tumor/saliva DNAs (26). This result may be supportive evidence as a comparative analysis with tumor tissue to address the heterogeneity of these markers. Ultimately, aberrant hypomethylation may be derived from cells that are from HNSCC, associated premalignancy, or histologically normal cells with epigenetic alterations. The definition of this phenomenon is beyond the scope of this investigation, but the prognostic significance of these data remains regardless of the underlying source of aberrant methylation.

The issue of specimen cellular representation on the interpretation and the potential application of these markers remains an important topic for investigation. In principle, differing methods of specimen collection may impact the representation of epigenetically altered cells in salivary rinses. We recently compared the promoter methylation pattern of these markers in 57 paired salivary rinses collected with or without an exfoliated brush from same patients with HNSCC (Unpublished data). Our study demonstrated strong correlations of gene promoter hypermethylation between salivary rinses collected with and without an exfoliating brush, suggesting that salivary rinse collected with and without exfoliating brush from patients with HNSCC share similar hypermethylation patterns.

In summary, our study investigated the prognostic significance of seven promoter methylation-based salivary rinse biomarkers in a large separately collected cohort of HNSCC patients based on a pilot study we reported previously. Our univariate analysis suggested that the detection of promoter hypermethylation of CCNA1, MGMT or MINT31 in salivary rinses is significantly associated with overall survival of HNSCC patients; the detection of promoter hypermethylation of MINT31 is significantly associated with disease-free survival; but none of them remained as independent prognostic factors in multivariate analysis. However, to the first time, we demonstrated that the detection of promoter hypermethylation of TIMP3 in salivary rinse is an independent prognostic factor of local recurrence overall survival. This has implication for directing specialized health care resources toward surveillance of patients with previously treated HNSCC who are at risk for recurrence, resulting in more efficient, improved surveillance and improved outcomes for patients at risk for recurrence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TRANSLATIONAL RELEVANCE

Head and Neck Squamous Cell Carcinoma (HNSCC) is a disease with significant morbidity and mortality. In this study the prognostic significances of seven epigenetic, promoter methylation-based salivary rinse biomarkers that we previously developed for HNSCC detection and surveillance were evaluated in a large, independent, prospectively collected cohort of HNSCC patients. We demonstrated detection of TIMP3 promoter hypermethylation in pre-treatment salivary rinse as an independent prognostic biomarker for local recurrence-free survival. Such a test could potentially refine our ability to identify HNSCC patients at a high risk for recurrence.

Figure 1. Promoter methylation levels for seven genes (P16, CCNA1, DCC, TIMP3, MGMT, DAPK and MINT31) in the DNAs from salivary rinses collected from 185 HNSCC cancer patients

The quantity of methylated allele of each gene was expressed as the ratio of the amount of polymerase chain reaction products amplified from the methylated gene to the amount amplified from the reference gene β actin multiplied by 100.

Figure 2. Cumulative incidence of local recurrence in 185 patients with HNSCC according to the promoter methylation status in salivary rinse

Table I
Baseline Characteristics of the HNSCC Patients (N=197)

Characteristic	No. of Patients	%
Age at study entry		
<55 yr	71	36.0%
55-64 yr	62	31.5%
>64 yr	64	32.5%
Median (year)	58	
Range	25 - 87	
Sex		
Male	150	76.1%
Female	47	23.9%
Race		
Caucasian	170	86.3%
African American	21	10.7%
Asian	4	2.0%
Smoking status (continuous for at least one year)		
No	54	27.4%
Yes	132	67.0%
Unknown	11	5.6%
Alcohol		
Never Used	39	19.8%
Used	133	67.5%
Unknown	25	12.7%
HPV		
Negative	28	14.2%
Positive	88	44.7%
Unknown	81	41.1%
Primary site		
Oral cavity	53	26.9%
Oropharynx	102	51.8%
Larynx	27	13.7%
Hypopharynx	6	3.0%
Unknown	9	4.6%
Pathological tumor stage		
T1	70	35.5%
T2	55	27.9%
T3	32	16.2%
T4	31	15.8%
Tx	9	4.6%
Pathological nodal stage		
N0	62	31.5%

Characteristic	No. of Patients	%
N1	18	9.1%
N2A	18	9.1%
N2B	78	39.6%
N2C	21	10.7%
Clinical TNM stage		
I	25	12.7%
II	18	9.1%
III	24	12.2%
IV	130	66.0%
Margin status		
Negative	64	32.5%
Dysplasia	11	5.6%
Cancer	64	32.5%
Primary tumor not removed	58	29.4%
Primary Therapy		
Surgery	153	77.7%
Radiation therapy	6	3.0%
Chemoradiotherapy	29	14.7%
Other/Combination Recurrence	9	6.6%
No	160	81.2%
Yes	36	18.3%
Missing	1	0.5%
Disease status at last FU		
No evidence of disease	131	66.5%
Alive with disease	6	3.0%
Died of disease	34	17.3%
Died of other causes	26	13.2%

Table II
Results of Univariate Analysis of Selected Prognostic Factors for Overall Survival, Disease-Free Survival, and Local Recurrence-Free Survival

	Overall Survival		Disease-Free Survival		Local Recurrence-Free Survival	
	HR	95% CI	HR	95% CI	HR	95% CI
Methylation Markers						
P16	1.82	[0.78, 4.26]	1.53	[0.70, 3.34]	1.12	[0.28, 4.48]
CCNA1	1.92	[0.99, 3.72]	1.72	[0.94, 3.15]	0.54	[0.13, 2.31]
DCC	1.43	[0.79, 2.58]	1.43	[0.86, 2.41]	1.30	[0.56, 3.03]
TIMP3	1.01	[0.49, 2.07]	1.33	[0.75, 2.36]	2.61	[1.16, 5.84]
MGMT	1.93	[1.10, 3.39]	1.57	[0.93, 2.64]	1.77	[0.77, 4.07]
DAPK	1.30	[0.55, 3.02]	0.95	[0.41, 2.20]	1.48	[0.46, 4.76]
MINT31	4.42	[1.88, 10.41]	2.86	[1.23, 6.67]	1.92	[0.44, 8.40]
Any marker positive	1.24	[0.74, 2.08]	1.28	[0.81, 2.04]	1.82	[0.81, 4.06]
Other risk factors						
Age	1.06	[1.04, 1.09]	1.05	[1.03, 1.07]	1.04	[1.00, 1.07]
Gender	1.02	[0.55, 1.90]	0.79	[0.47, 1.33]	0.65	[0.29, 1.48]
Smoking status						
Yes vs. No	1.93	[1.0, 3.75]	1.51	[0.87, 2.64]	1.57	[0.64, 3.86]
Stage						
III/IV vs. I/II	1.27	[0.67, 2.41]	0.94	[0.55, 1.58]	0.60	[0.28, 1.29]
HPV	0.30	[0.16, 0.57]	0.35	[0.20, 0.60]	0.64	[0.24, 1.21]
Primary tumor site						
Oropharynx vs. other	0.39	[0.22, 0.67]	0.43	[0.27, 0.70]	0.45	[0.21, 1.01]
Pathological tumor stage						
T3/T4 vs. T1/T2	4.07	[2.39, 6.92]	2.77	[1.75, 4.39]	1.46	[0.66, 3.20]
Pathological nodal stage						
N1/N2 vs. N0	1.01	[0.59, 1.72]	0.81	[0.51, 1.29]	0.74	[0.35, 1.54]
Margin status						
Cancer vs. other	1.83	[1.08, 3.10]	1.46	[0.90, 2.36]	1.28	[0.59, 2.78]

Table III
Results of Multivariate Analysis of Selected Prognostic Factors for Overall Survival, Disease-Free Survival, and Local Recurrence-Free Survival

Marker	Overall Survival [¶]		Disease-Free Survival [§]		Local Recurrence-Free Survival [‡]	
	HR	95% CI	HR	95% CI	HR	95% CI
PI6	0.79	[0.31, 1.99]	0.96	[0.43, 2.15]	0.90	[0.20, 4.01]
CCNA1	0.96	[0.48, 1.93]	0.93	[0.49, 1.74]	0.45	[0.10, 2.03]
DCC	0.79	[0.42, 1.50]	0.97	[0.56, 1.67]	1.13	[0.45, 2.83]
TIMP3	1.77	[0.82, 3.82]	1.69	[0.92, 3.11]	2.51	[1.10, 5.68]
MGMT	0.91	[0.47, 1.75]	0.96	[0.53, 1.73]	1.62	[0.69, 3.82]
DAPK	2.19	[0.84, 5.71]	1.42	[0.59, 3.43]	1.41	[0.44, 4.53]
MINT31	2.26	[0.85, 6.05]	1.77	[0.71, 4.38]	1.66	[0.35, 7.84]
Any marker positive	0.95	[0.53, 1.69]	1.06	[0.64, 1.76]	1.63	[0.70, 3.79]

[¶]For overall survival endpoint, the multivariable model included age, HPV, primary site, pathological T stage and margin status as covariates. Detailed multivariate analysis of these covariates was in Supplementary Table I.

[§]For disease-free survival endpoint, the multivariable model included age, HPV, primary site and T stage as covariates. Detailed multivariate analysis of these covariates was in Supplementary Table II.

[‡]For local recurrence-free survival endpoint, the multivariable model included primary site as a covariate. Detailed multivariate analysis of these covariates was in Supplementary Table III.