Evaluation of Promoter Hypermethylation Detection in Body Fluids as a Screening/Diagnosis Tool for Head and Neck Squamous Cell Carcinoma

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Abstract

Purpose: To evaluate aberrant promoter hypermethylation of candidate tumor suppressor genes as a means to detect epigenetic alterations specific to solid tumors, including head and neck squamous cell carcinoma (HNSCC).

Experimental Design: Using promoter regions identified via a candidate gene and discovery approach, we evaluated the ability of an expanded panel of CpG-rich promoters known to be differentially hypermethylated in HNSCC in detection of promoter hypermethylation in serum and salivary rinses associated with HNSCC. We did preliminary evaluation via quantitative methylation-specific PCR (Q-MSP) using a panel of 21 genes in a limited cohort of patients with HNSCC and normal controls. Using sensitivity and specificity for individual markers as criteria, we selected panels of eight and six genes, respectively, for use in salivary rinse and serum detection and tested these in an expanded cohort including up to 211 patients with HNSCC and 527 normal controls.

Results: Marker panels in salivary rinses showed improved detection when compared with single markers, including a panel with 35% sensitivity and 90% specificity and a panel with 85% sensitivity and 30% specificity. A similar pattern was noted in serum panels, including a panel with 84.5% specificity with 50.0% sensitivity and a panel with sensitivity of 81.0% with specificity of 43.5%. We also noted that serum and salivary rinse compartments showed a differential pattern of methylation in normal subjects that influenced the utility of individual markers.

Conclusions: Q-MSP detection of HNSCC in serum and salivary rinses using multiple targets offers improved performance when compared with single markers. Compartment-specific methylation in normal subjects affects the utility of Q-MSP detection strategies.

There are >40,000 new cases of head and neck squamous cell carcinoma (HNSCC) in the United States each year, with a mortality rate of 12,000 U.S. deaths annually. These incidence and mortality figures correspond to >4% of all new cancer cases and 2% of all cancer deaths in the United States each year. There have been modest improvements in survival for patients with HNSCC in the past 30 years. From 1995 to 2001, only ~30% of the HNSCC in the Unites States have been diagnosed at an early clinical stage (1). Intuitively, early detection of HNSCC would improve clinical outcomes. Currently, there is no definitive evidence that widespread population screening using routine head and neck examination with or without fiberoptic endoscopy and/or vital staining would result in a decrease in mortality from HNSCC (2). However, there is evidence that screening high-risk populations may be cost effective (3).

The use of molecular markers in body fluids in molecular detection has been explored with the intent to improve screening accuracy and cost-effectiveness. 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. Examples of relevant body fluids used for detection include analysis of sputum for lung cancer diagnosis (4), urine for urologic tumors (5), saliva for HNSCC (6–8), breast fluid (9), as well as serum or plasma for almost all types of cancer (10–14).

An epigenetic pathway of transcriptional inactivation for many tumor suppressor genes includes CpG island hypermethylation

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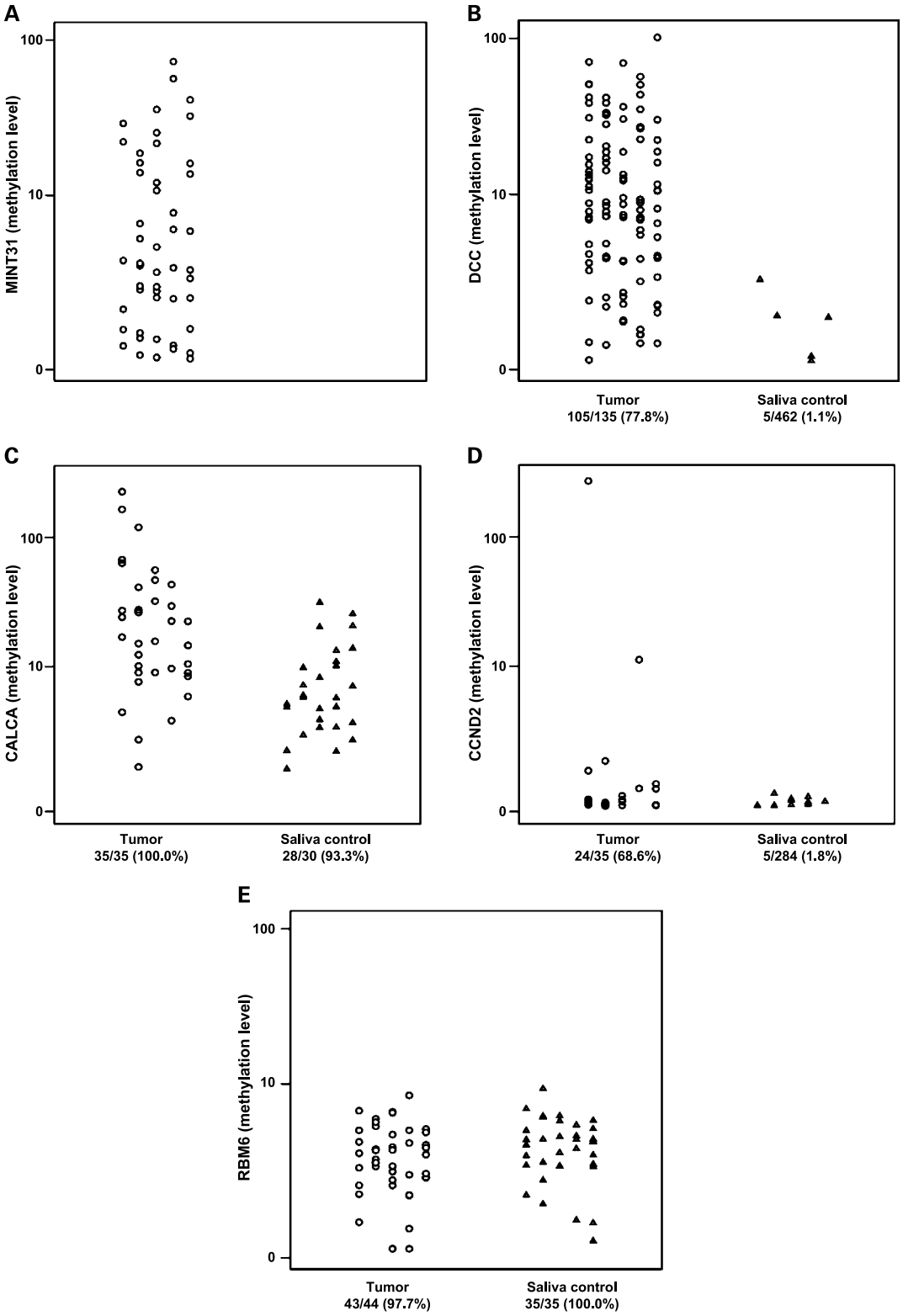

Fig. 1. Patterns of hypermethylation observed in DNA tumor (case) and DNA salivary rinses (control). *A,* methylation was detected only in HNSCC but not in controls. *B,* a higher frequency and quantity of methylation was noted in HNSCC compared with controls with absent methylation in a subset of control samples. *C,* a higher frequency of methylation was noted in HNSCC compared with controls but in a similar quantity in both groups. *D,* a similar frequency of methylation was noted in both groups (tumor and salivary rinses); however, a quantitative difference between groups was noted. *E,* methylation was noted in both HNSCC and controls at a similar frequency with no difference in methylation quantity. *X* axis, proportion of methylated cases/tested cases for each sample type; *Y* axis, quantity of hypermethylation (gene of interest/ACTB $\times$ 100).

within promoter regions (15–17). This pathway has been identified in many different cancers and recent studies have focused on promoter hypermethylation in HNSCC (18, 19). Promoter hypermethylation in tissue samples can be detected by using quantitative methylation-specific PCR (Q-MSP); this real-time PCR methodology allows to a more objective, robust, and rapid assessment of promoter methylation status. The ability to quantify the methylation provides the potential for determination of a threshold value of methylation to improve sensitivity and specificity in detection of tumor-specific signal (20–23).

The detection of DNA methylation in body fluids also has the potential to distinguish high-risk subjects that either harbor occult cancers or have a higher risk for development of cancer. Palmisano et al. (4) were able to detect aberrant promoter methylation in sputum of patients with squamous cell lung carcinoma up to 3 years before clinical diagnosis. Subsequently, using a panel of genes, they were able to identify patients at high risk for cancer incidence by detecting DNA hypermethylation in sputum in a prospective study (24). Identification of differential promoter hypermethylation patterns between primary tumors and saliva or serum obtained from patients with HNSCC has already been shown in limited cohorts with a limited number of genes (7, 12, 25–27).

We did this study to determine if an expanded panel of promoter hypermethylation markers would result in an improved ability to detect epigenetic changes associated with HNSCC in salivary rinses and serum from patients with HNSCC. During this study, we were able to show differential promoter hypermethylation in HNSCC patients compared with normal individuals in these body fluid compartments.

Materials and Methods

Tissue samples. Samples from 211 HNSCC patients were obtained from patients presenting a previously untreated squamous cell carcinoma from the oral cavity, larynx, or pharynx. Patients were evaluated and enrolled in appropriate protocols in the Department of Otolaryngology-Head and Neck Surgery at Johns Hopkins Medical Institutions (Baltimore, MD) using appropriate informed consent obtained after institutional review board approval. Tumor, salivary rinse, and serum samples from these patients were collected.

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 tissue collected using this technique includes exfoliated epithelial cells from the upper aerodigestive tract, and an exfoliating brush is used to include cells from deep epithelial layers in the oral cavity and oropharynx. This technique allows for a broad sampling of epithelial cells from multiple sites in the upper aerodigestive tract. 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. Cellular material from the brushing was released into the saline rinse and centrifuged to obtain a cell pellet after supernatant was discarded.

The control population consists of subjects enrolled in a community screening study for head and neck cancer approved by the Johns Hopkins institutional review board. The experimental protocol was approved by the Johns Hopkins Medical Institutions Institutional Review Board and informed consent was obtained from all enrolled subjects. For the control population, a salivary rinse and blood sample were collected. All subjects were administered a confidential written survey of risk factors for upper aerodigestive tract malignancies, including alcohol and tobacco use as well as the presence of comorbid illnesses. Smoking was defined as use of tobacco, chewable or smoked, for at least 1 year continuously. Heavy alcohol use was defined as intake of more than two alcoholic drinks per day. A thorough head and neck examination, including cranial nerve function; palpation of cervical, thyroid, and parotid nodal basins; visual inspection and palpation of the oral cavity and oropharynx; and indirect mirror laryngoscopy or flexible fiberoptic laryngoscopy, was done.

Of those participants initially enrolled in the screening study, five have dropped out due to inability to obtain blood samples. There have been no adverse events reported. All individuals were called by phone once a year afterwards and interviewed to determine interval changes in tobacco and alcohol consumption and health history, including new cancer diagnosis. For the panel analysis, we excluded those individuals presenting with premalignant or malignant lesions at head and neck area (n = 33), past history of cancer regardless of site (n = 57), those who were diagnosed of any cancer regardless of site during follow-up (n = 62), and those not reachable by phone follow-up (n = 119). A total of 527 individuals were included as control population in this study.

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

Bisulfite treatment. DNA from tissue samples was subjected to bisulfite treatment as described previously (28). Briefly, 2 μg of genomic DNA were denatured in 0.2 mol/L NaOH for 20 min at 50°C. The denatured DNA was diluted in 500 μL of freshly prepared solution of 10 mmol/L hydroquinone and 3 mol/L sodium bisulfite and incubated for 3 h at 70°C. After incubation, the DNA sample was desalted through a column (Wizard DNA Clean-Up System, Promega), treated with 0.3 mol/L NaOH for 10 min at room temperature, and precipitated overnight with ethanol. The bisulfite-modified genomic DNA was resuspended in 120 μL H_2O and stored at -80°C.

Quantitative methylation-specific PCR. The bisulfite-modified DNA was used as a template for fluorescence-based real-time PCR as described previously (29). In brief, primers and probes were designed to specifically amplify the bisulfite-converted DNA for the *ACTB* gene and all genes of interest (primers and probes sequences are available on Supplementary Table S1). The ratios between the values of the gene of interest and the internal reference gene (*ACTB*), which was obtained by Taqman analysis and take into account the PCR efficiency, were used as a measure for representing the relative quantity of methylation in a particular sample (value for the gene of interest/value for the reference gene × 100). Fluorogenic PCRs were carried out in a reaction volume of 20 μL consisting of 600 nmol/L of each primer; 200 μmol/L of probe; 0.75 unit of platinum Taq polymerase (Invitrogen); 200 μmol/L of each dATP, dCTP, dGTP, and dTTP; 200 nmol/L of ROX Reference Dye (Invitrogen); 16.6 mmol/L ammonium sulfate; 67 mmol/L Trizma (Sigma); 6.7 mmol/L magnesium chloride; 10 mmol/L mercaptoethanol; and 0.1% DMSO. Three microliters of treated DNA solution were used in each real-time MSP reaction. 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 min followed by 45 cycles of 95°C for 15 s and 60°C or 62°C for 1 min. Leukocytes from a healthy individual were methylated *in vitro* with excess *Sss*I methyltransferase (New England Biolabs) to generate completely methylated DNA, and serial dilutions of this DNA were used for constructing the calibration curves on each plate. Each reaction was done in triplicate; the average of the triplicate was considered for analysis. Results for Q-MSP was analyzed considering the quantity of methylation (normalized by ACTB) as well as considering methylation as a binary event, in which any quantity of methylation in a sample would be considered as positive for methylation.

Target gene selection. Genes selected for this study came from three different sources: (*a*) genes with promoters that are reported as

Table 1. Comparison of hypermethylation detection on tumor DNA (HNSCC patients) and salivary rinse samples (controls)

Gene	Tumor, case (*n*)	Salivary rinses, control (*n*)	*P**	Sensitivity, % (95% CI)	Specificity, % (95% CI)
DCC	135	462	<0.0001	77.8 (69.8-84.5)	98.9 (97.5-99.7)
DAPK	136	451	<0.0001	75.0 (66.9-82.0)	96.2 (94.0-97.8)
TIMP3	138	450	<0.0001	73.9 (65.8-81.0)	92.9 (90.1-95.1)
ESR	35	119	<0.0001	60.0 (42.1-76.1)	98.3 (94.1-99.8)
CCNA1	102	444	<0.0001	61.8 (51.6-71.2)	97.1 (95.1-98.4)
CCND2	35	97	<0.0001	68.6 (50.7-83.2)	89.7 (81.9-94.7)
MINT1	87	296	<0.0001	90.8 (82.7-96.0)	66.2 (60.5-71.6)
MINT31	136	492	<0.0001	36.8 (28.7-45.5)	100 (99.4-100)
CDH1	77	116	<0.0001	90.9 (82.2-96.3)	37.9 (29.1-47.4)
AIM1	77	73	<0.0001	23.4 (14.5-34.4)	98.6 (92.6-100)
MGMT	44	239	<0.0001	22.7 (11.5-37.8)	95.0 (91.4-97.4)
p16	136	500	<0.0001	13.2 (8.0-20.1)	100 (99.4-100)
PGP9.5	45	112	0.004	91.1 (78.8-97.5)	30.4 (22.0-39.8)
RARB	44	35	0.005	79.6 (64.7-90.2)	51.4 (34.0-68.6)
HIC1	45	46	0.026	100 (93.6-100)	13.0 (4.9-26.3)
RASSF1A	44	35	0.063	11.4 (3.8-24.6)	100 (91.8-100)
CALCA	35	30	0.209	100 (91.8-100)	6.7 (0.8-22.1)
TGFBR2	11	44	0.266	54.6 (23.4-83.3)	25.0 (13.2-40.3)
S100A2	44	35	0.398	95.5 (84.5-99.4)	11.4 (3.2-26.7)
RIZ1	44	35	0.694	6.8 (1.4-18.7)	88.6 (73.3-96.8)
RBM6	44	35	>0.999	97.7 (88.0-99.9)	0 (0-8.2)

*Fisher's exact test for the association between gene methylation and HNSCC.

hypermethylated in HNSCC [*DCC, p16(INK4A), CDH1, MGMT, DAPK, RASSF1A, RARB*, and *RIZ1*]; also including two genome regions known to have a differentially methylated pattern in some tumors (MINT1 and MINT31; refs. 30–34); (*b*) genes with promoters that are reported as hypermethylated in other solid tumors (*CCND2, CALCA, TGFBR2, HIC1, S100A2, TIMP3*, and *ESR*; refs. 9, 35–38); and (*c*) gene discovery using expression microarray-based approach via unmasking of expression (*CCNA1, PGP9.5, AIM1*, and *RBM6*; ref. 39).

Steps for gene evaluation in the study. Due to the scarcity of DNA quantity after bisulfite treatment of many samples and the number of genes selected, it would be virtually impossible to evaluate all possible candidate genes in all samples (the exact number of cases considered for each analysis is described on Supplementary Tables S2 and S3). We decided to do a step by step selection with interim statistical analysis, and then a more limited set of "best" genes would be used in an expanded cohort of samples. The first step involved a screening evaluation, designed to eliminate targets that had an inappropriately high frequency of promoter hypermethylation in normal, control samples. Elimination of these targets with high rates of promoter hypermethylation in normal control samples facilitated efficient use of limited sample material and allowed for an early definition of higher value markers. We did a screening evaluation by evaluating candidate genes by comparing tumor samples (cases) with salivary rinses or serum (from controls) in a limited, random subset of both the patient and controls. We also did a screening evaluation directed at the serum and salivary rinse compartments by comparison between salivary rinses (case) from salivary rinses (control) and serum (case) from serum (control) in additional limited sets of HNSCC patients and controls. For this first step of interim analysis, we used a subset of samples (those with higher concentration of DNA) from the complete cohort. Significance was based on area under the curve (AUC) from receiving operating characteristic analysis, sensitivity, and specificity of that particular gene in differentiating the tumor samples (cases) from salivary rinses or serum (controls) for determination of a salivary rinse marker panel, with similar criteria when differentiating salivary rinses (case) from salivary rinses (control) or serum (case) from serum (control).

Markers that fit selection criteria in initial analysis (see Results) were then used in an expanded analysis of all available tissue from HNSCC patient cases and control subjects. Complete coverage of every sample for every possible methylation marker was not possible due to either limited sample collection or a low quantity of total extracted DNA (particularly for a small proportion of serum samples).

Statistical analysis. A total of 21 informative genes were considered for this study. Hypermethylation of each gene was treated as a binary variable (methylation versus no methylation) by dichotomizing each gene at zero. Proportions of gene methylation were compared between tumor samples (from cases) and salivary rinses or serum samples (from controls) using Fisher's exact test. Sensitivity and specificity of each individual gene in detecting HNSCC were calculated along with 95% confidence intervals (95% CI), where sensitivity was defined as the number of true-positive results divided by the number of true-positive plus false-negative results and specificity was defined as the number of true-negative results divided by the number of true-negative plus false-positive results. We evaluated all possible combinations of the selected markers for both saliva and serum samples in the expanded cohort, where a positive panel was defined as at least one gene of the panel being methylated (Supplementary Tables S2 and S3). Sensitivity and specificity were calculated along with 95% CIs. The AUC, an index of predictive power, was also provided. The potential of the confounding effect of the covariates, including age, gender, and tobacco and alcohol assumption, was assessed using stratified analysis. In the meantime, the performances of the selected panels were explored using multivariable logistic regression method. A receiving operating characteristic curve was constructed by plotting the sensitivity (true-positive rate) against 1-specificity (false-positive rate). Internal validation of the logistic regression models was done by using an approximation to the leave-one-out jackknife procedure implemented with Statistical Analysis System software package. Receiving operating characteristic curves for some selected panels based on the method of multivariable logistic regression modeling were constructed for both salivary rinses and serum samples, where the single point represented the performance of the panel with a positive panel being defined as at least one gene of the panel presented methylation

(Supplementary Figs. S1 and S2). All statistical tests were two sided with $P \leq 0.05$ considered statistically significant. All analyses were done using Statistical Analysis System software (version 9.1; SAS Institute, Inc.) and R package (version 1.9.1).

Results

HNSCC patients and control population characteristics. HNSCC patients were mainly males (75.5%) and Caucasians (78.1%), with ages ranging from 32 to 89 years (median, 57.8 years). Alcohol or tobacco consumption (current or past) was found in 71.3% and 81.7%, respectively.

The control population (subjects enrolled in a community screening study) was mainly males (59.6%) and Caucasians (78.6%), with ages ranging from 18 to 94 years (median, 61.0 years). Alcohol or tobacco consumption (current or past) was found in 79.8% and 61.8%, respectively.

Initial screening: tumor (case) versus salivary rinses (control). As a first step, we evaluate the specificity and sensitivity comparing the presence of promoter hypermethylation of selected genes in tumor DNA (from cases) and salivary rinse DNA (from controls). Although these are not identical tissues, we used this method because (*a*) discovered targets would be subjected to additional validation using a second comparison of salivary rinses from both patient cohorts, (*b*) our collection method used an exfoliating brush that removes deeper layers of oral and oropharyngeal mucosa, and (*c*) formal biopsy of the >400 noncancer patients was not logistically feasible. We could note distinct methylation patterns as follows: (*a*) methylation was detected only in HNSCC but not in controls: *p16*, *MINT31*, and *RASSF1A*; (*b*) a higher frequency and quantity of methylation was noted in HNSCC compared with controls with absent methylation in a subset of control samples: *DCC*, *DAPK*, *CCNA1*, *TIMP3*, *MGMT*, *AIM1*, *ESR*, *MINT1*, *CDH1*, *RARB*, *PGP9.5*, and *HIC1*; (*c*) a higher frequency of methylation was noted in HNSCC compared with controls but in a similar quantity in both groups: *CCND2*; (*d*) a similar frequency of methylation was noted in both groups (tumor and salivary rinses); however, a quantitative difference between groups was noted: *CALCA*; and (*e*) methylation was noted in both HNSCC and controls at a similar frequency with no

Table 2. Comparison of hypermethylation detection on salivary rinse samples (HNSCC patients) and salivary rinse samples (controls)

A. Individual gene evaluation

Gene	Case (*n*)	Control (*n*)	Sensitivity, % (95% CI)	Specificity, % (95% CI)
CCNA1	175	444	20.0 (14.3-26.7)	97.1 (95.1-98.4)
DAPK	176	451	15.9 (10.8-22.2)	96.2 (94.0-97.8)
DCC	176	462	11.9 (7.5-17.7)	98.9 (97.5-99.7)
MGMT	149	239	13.4 (8.4-20.0)	95.0 (91.4-97.4)
TIMP3	176	450	11.4 (7.1-17.0)	92.9 (90.1-95.1)
MINT31	175	492	4.6 (2.0-8.8)	100.0 (99.4-100)
p16	177	500	4.5 (2.0-8.7)	100.0 (99.4-100)
PGP9.5	34	112	82.4 (65.5-93.2)	30.4 (22.0-39.8)
AIM1	23	73	4.4 (0.1-22.0)	98.6 (92.6-100)
ESR	33	119	3.0 (0.08-15.8)	98.3 (94.1-99.8)
CCND2	136	97	7.4 (3.6-13.1)	89.7 (81.9-94.9)
MINT1	131	296	35.1 (27.0-43.9)	66.2 (60.5-71.6)
CDH1	66	116	30.3 (19.6-42.9)	37.9 (29.1-47.4)

B. Best combination of genes for hypermethylation HNSCC detection on salivary rinses

Panel	Case (*n*)	Control (*n*)	Sensitivity, % (95% CI)	Specificity, % (95% CI)
CCNA1 + DCC + DAPK + p16	176	417	33.5 (26.6-41.0)	91.8 (88.8-94.3)
MINT31 + CCNA1 + DCC + DAPK + p16	176	417	34.1 (27.1-41.6)	91.8 (88.8-94.3)
MINT31 + MGMT + CCNA1 + p16	151	240	35.1 (27.5-43.3)	90.0 (85.5-93.5)
MINT31 + CCNA1 + DCC + p16	175	444	28.6 (22.0-35.9)	95.9 (93.7-97.6)
CCNA1 + DCC + p16	175	444	28.0 (21.5-35.3)	95.9 (93.7-97.6)
MINT31 + CCNA1 + DAPK + p16	176	416	30.7 (24.0-38.1)	92.8 (89.9-95.1)
CCNA1 + DCC + DAPK	176	417	31.8 (25.0-39.3)	91.8 (88.8-94.3)
MINT31 + CCNA1 + DCC + DAPK	175	417	33.1 (26.2-40.6)	91.8 (88.8-94.3)
MINT31 + MGMT + CCNA1	150	240	34.7 (27.1-42.9)	90.0 (85.5-93.5)
MGMT + CCNA1 + p16	151	240	33.8 (26.3-41.9)	90.0 (85.5-93.5)
MGMT + CCNA1	150	240	33.3 (25.9-41.5)	90.0 (85.5-93.5)
CCNA1 + DCC	175	444	25.7 (19.4-32.9)	95.9 (93.7-97.6)
MINT31 + CCNA1 + DCC	174	444	27.0 (20.6-34.3)	95.9 (93.7-97.6)
MINT31 + CCNA1 + DAPK	175	416	29.7 (23.1-37.1)	92.8 (89.9-95.1)
CCNA1 + DAPK + p16	176	416	29.5 (22.9-36.9)	92.8 (89.9-95.1)
MINT31 + CCNA1 + p16	175	444	24.0 (17.9-31.0)	97.1 (95.1-98.4)
CCNA1 + DAPK	176	416	27.8 (21.4-35.1)	92.8 (89.9-95.1)
MINT31 + CCNA1	174	444	22.4 (16.5-29.3)	97.1 (95.1-98.4)
CCNA1 + p16	175	444	22.3 (16.4-29.2)	97.1 (95.1-98.4)
DCC + DAPK + p16	176	422	24.4 (18.3-31.5)	95.0 (92.5-96.9)
MINT31 + DCC + DAPK + p16	176	422	25.0 (18.8-32.1)	95.0 (92.5-96.9)

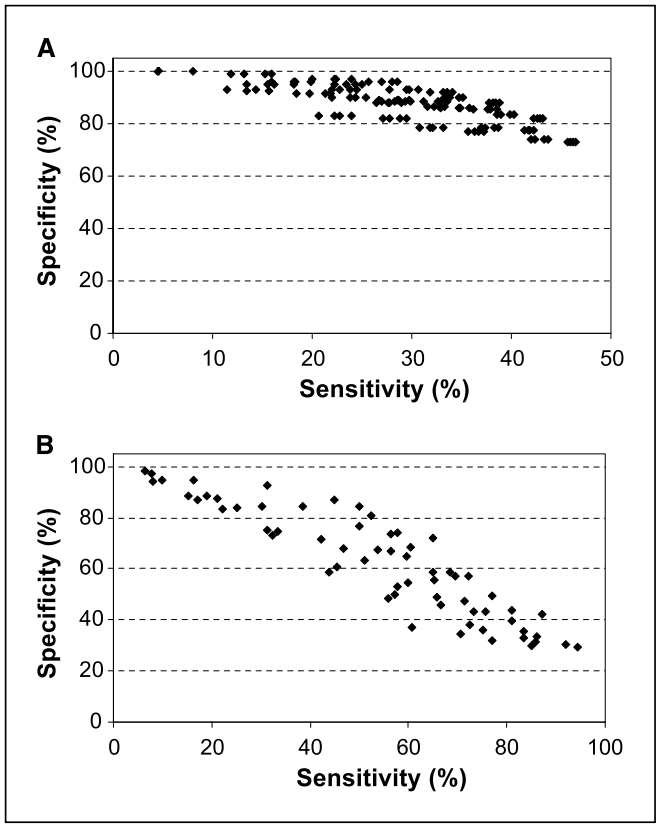

Fig. 2. Plot of specificity versus sensitivity for head and neck cancer detection on body fluids (graph representation of Supplementary Tables S2 and S3). *A,* plot for detection in salivary rinses considering all possible combination for the selected genes. *B,* plot for detection in serum considering all possible combination for the selected genes.

difference in methylation levels: *RIZ1*, *TGFBR2*, *S100A2*, and *RBM6*. Examples of these patterns are shown on Fig. 1, with overall results shown in Table 1. We were able to define 12 genes whose methylation was highly associated with HNSCC, with local recurrences ranging from 1.1 to 70.7.

Initial screening: salivary rinse (case) versus salivary rinse (control). Based on the above results, genes that could distinguish tumor samples (case) from salivary rinse samples (control) for binary results (either presence or absence of methylation) and an AUC >0.60 and at least 90% specificity or sensitivity were selected for further testing on salivary rinses in a limited cohort of HNSCC patients. These markers included *CCNA1*, *DAPK*, *DCC*, *MGMT*, *TIMP3*, *MINT31*, *p16*, *PGP9.5*, *AIM1*, *ESR*, *CCND2*, *MINT1*, and *CDH1*. We observed that, in general, although some genes were quite specific for HNSCC compared with DNA from salivary rinses (controls), the sensitivity of these makers for detection of HNSCC in the salivary rinses of HNSCC patients was quite low (Table 2A). We again noted that seven genes now showed local recurrences associated with HNSCC, with ranges from 1.2 to 10.8.

HNSCC detection in salivary rinses in expanded cohort. Based on the above results, the genes that could better distinguish the HNSCC patient salivary rinse from control salivary rinse, with an AUC >0.50 and at least 90% specificity and 10% sensitivity, were selected to be tested in combination for the expanded cohort. We also tested *MINT31* and *p16*, as both markers were 100% specific. The results for AUC, sensitivity, and specificity for all possible combinations with the selected genes are included in Supplementary Table S2, and the plot for sensitivity and specificity for those combinations is shown on Fig. 2A. The best performing combination panels are presented in Table 2B, and we observed that some panels can show up to 91.8% specificity with 34.1% sensitivity. Specificity can be observed as high as 97.1% in some combination of genes; however, for these combinations, sensitivity ranges from 22.3% to 24.0%.

Initial screening: tumor (case) versus serum (control). Again, as a first step in developing a detection panel for serum, we evaluated specificity and sensitivity by comparing promoter hypermethylation of selected genes in tumor DNA versus serum DNA from control subjects without cancer. We noted distinct methylation patterns as follows: (*a*) hypermethylation was detected only in HNSCC but not in controls: *p16*, *MINT31*, *CCNA1*, *DCC*, *RARB*, *ESR*, *AIM1*, and *RIZ1*; (*b*) a higher frequency and quantity of methylation was noted in HNSCC compared with controls: *TGFBR2*, *TIMP3*, *CDH1*, *MINT1*, *RBM6*, and *CALCA*; (*c*) a higher frequency of methylation was noted in HNSCC with no difference in methylation levels: *RASSF1A*, *PGP9.5*, *HIC1*, *DAPK*, and *CCND2*; and (*d*) a similar frequency of methylation was noted in both groups; however, a quantitative difference was noted: *S100A2*. Overall results are shown on Table 3. Fourteen genes were associated with HNSCC with local recurrences ranging from 2.9 to 38.1.

Initial screening: serum (case) versus serum (control). Based on the above results, the genes that could distinguish the tumor samples (case) from serum samples (control) using binary results (presence or absence of methylation) and AUC >0.60 and at least 90% specificity or sensitivity were selected to be tested on serum from the HNSCC patients. Despite the fact that *MINT1* and *RBM6* would fulfill these criteria, the levels of methylation observed in the previous analysis showed higher levels of methylation in control serum than in tumor DNA, so these two markers were excluded as candidates for the panel. We then selected *CCNA1*, *DCC*, *TIMP3*, *MINT31*, *p16*, *PGP9.5*, *AIM1*, *ESR*, *CCND2*, *CDH1*, *TGFBR2*, and *HIC1*. Of note, we could observe, again, that despite the fact that some genes were quite specific for HNSCC compared with DNA from serum (controls), the sensitivity of these genes to be detected in the serum from the HNSCC patients was quite low or undetectable (Table 4A). Six genes were found to be significantly associated with HNSCC.

HNSCC detection in serum in an expanded cohort. Based on the above results, genes that could better distinguish the HNSCC patient salivary rinses from control salivary rinses, presenting an AUC >0.50 and at least 90% specificity and 10% sensitivity, were selected to be tested in combination for the expanded cohort. Based on these criteria, we selected three genes; however, a sufficient quantity of serum DNA allowed for evaluation of six genes. Therefore, we decided to include *CDH1* (due to its high sensitivity), *CCND2*, and *TGFBR2*. The results for AUC, sensitivity, and specificity for all possible combination with the selected genes are on Supplementary Table S3, and the plot for sensitivity and specificity for these combinations is shown in Fig. 2B. The most favorable combinations are presented in Table 4B and show that some panels provide up to 84.5% specificity with 50.0% sensitivity and that specificity can be observed as high as 92.5% in one combination of genes; however, sensitivity for this combination is only 31.4%. For

other marker combinations, we could observe sensitivity as high as 81.0%; however, the corresponding specificity was 43.5%.

Compartment-specific hypermethylation. We noted that some markers exhibited significant presence in normal control subject sera and salivary rinses, although sometimes in only one compartment (Fig. 3). For example, *TIMP3* would show good specificity in distinguishing tumor samples from salivary rinses and serum control samples based on methylation results, allowing its use on both panels; on the other hand, S100A2 would not show any specificity for distinguishing groups based on salivary rinses and serum analysis. Some particular genes, such as *TGFBR2*, showed significant promoter methylation in salivary rinses from normal controls as well as tumor tissue from HNSCC patients but still showed a low frequency of methylation in sera from normal controls. This allows use of *TGFBR2* in a serum detection panel but prevents its use in detection of HNSCC in salivary rinses. Conversely, *DAPK* has shown methylation in serum from normal control patients as well as in primary HNSCC, consistent with previous findings (40), but only has minimal methylation in salivary rinses from control subjects, allowing for use in a salivary rinse detection panel. Other markers showed this phenomenon of compartment-specific methylation in normal controls (Fig. 3), and this phenomenon made a significant effect in the determination of separately constructed detection panels depending on the body fluid or cellular compartment of interest for detection.

Discussion

Aberrant promoter hypermethylation has been proposed as a means for detection of tumor-specific cells in body fluids and exfoliated cells in solid tumors, including HNSCC. We have studied a large sample size of both controls and HNSCC patients using an expanded panel of methylated promoter regions to determine the ability of Q-MSP to detect tumor-specific promoter methylation in serum and salivary rinses. We used salivary rinses obtained from rinses and brushing as a normal control tissue to obtain a broad representation of epithelial cells from the upper aerodigestive tract. Use of site-matched control tissues, for example, would ignore the possibility of site-specific contamination from other sites in the upper aerodigestive tract (e.g., lymphoepithelial contamination in the oropharynx) and was therefore not used. Given the sensitivity of the Q-MSP technique used to detect the presence of methylated alleles in a background of normal at a threshold of 1/1,000 to 1/10,000, this strategy allowed us to define methylated genes that were highly specific for tumor and rarely or never present in any of the aerodigestive sites that shed cells in salivary rinses. In this sense, our selection of a control tissue that is obtained in a manner similar to those that will likely be used in a surveillance or screening strategy provides an advantage in selecting appropriate targets in this analysis.

We were able to confirm the significant role of promoter methylation as a means of epigenetic alteration in HNSCC based on the determination that ~100% of the cases have shown methylation in the tumor DNA for at least one of the studied genes. This would indicate the potential for use of aberrant promoter hypermethylation as a tool for detection of HNSCC and reinforce the potential for use of this technology in screening and surveillance. We have also confirmed that detection of tumor-specific promoter hypermethylation is feasible in body fluids (4, 7, 12) and the Q-MSP is well adapted into a high-throughput format (20–23).

Some studies with limited cohorts have shown that promoter methylation was HNSCC specific and could not be detected in healthy controls in salivary rinse, mucosal cytobrush, or serum (30, 41). We were able to confirm an elevated frequency of promoter hypermethylation in HNSCC in a panel of gene

Table 3. Comparison of hypermethylation detection on tumor DNA (HNSCC patients) and serum samples (controls)

Gene	Tumor case (*n*)	Serum control (*n*)	*P**	Sensitivity, % (95% CI)	Specificity, % (95% CI)
HIC1	45	373	<0.0001	100 (93.6-100)	92.5 (89.3-95.0)
PGP9.5	45	203	<0.0001	91.1 (78.8-97.5)	97.5 (94.4-99.2)
TGFBR2	42	134	<0.0001	88.1 (74.4-96.0)	94.0 (88.6-97.4)
RARB	44	85	<0.0001	79.6 (64.7-90.2)	100 (96.5-100)
DCC	135	135	<0.0001	77.8 (69.8-84.5)	100 (97.8-100)
TIMP3	138	296	<0.0001	73.9 (65.8-81.0)	94.6 (91.4-96.9)
CCNA1	35	284	<0.0001	68.6 (51.6-71.2)	98.2 (97.2-100)
CDH1	77	320	<0.0001	90.9 (82.2-96.3)	73.1 (67.9-77.9)
CCND2	35	284	<0.0001	68.6 (50.7-83.2)	98.2 (95.9-99.4)
ESR	35	20	<0.0001	60.0 (42.1-76.1)	100 (86.1-100)
MINT1	87	19	<0.0001	90.8 (82.7-96.0)	68.4 (43.5-87.4)
MINT31	136	42	<0.0001	36.8 (28.7-45.5)	100 (93.1-100)
AIM1	77	41	<0.0001	23.4 (14.5-34.4)	100 (93.0-100)
p16	136	102	<0.0001	13.2 (8.0-20.1)	100 (97.1-100)
CALCA	35	20	0.005	100 (91.8-100)	25.0 (8.7-49.1)
RASSF1A	44	104	0.009	11.4 (3.8-24.6)	99.0 (94.8-100)
RBM6	44	18	0.022	97.7 (88.0-100)	22.2 (6.4-47.6)
S100A2	44	12	0.198	95.5 (84.5-99.4)	16.7 (2.1-48.4)
RIZ1	44	18	0.550	6.8 (1.4-18.7)	100 (84.7-100)

*Fisher's exact test for the association between gene methylation and HNSCC.

Table 4. Comparison of hypermethylation detection for single genes on serum samples (HNSCC patients) and serum samples (controls)

A. Individual gene evaluation

Gene	Case (*n*)	Control (*n*)	Sensitivity, % (95% CI)	Specificity, % (95% CI)
HIC1	70	373	31.4 (20.9-43.6)	92.5 (89.3-95.0)
PGP9.5	52	203	7.7 (2.1-18.5)	97.5 (94.4-99.2)
CDH1	62	320	32.3 (20.9-45.3)	73.1 (67.9-77.9)
CCND2	47	284	6.4 (1.3-17.5)	98.2 (95.9-99.4)
TIMP3	50	296	10.0 (3.3-21.8)	94.6 (91.4-96.9)
TGFBR2	37	134	8.1 (1.7-21.9)	94.0 (88.6-97.4)
AIM1	10	41	10.0 (0.3-44.5)	100 (93.0-100)
ESR	16	20	6.3 (0.2-30.2)	100 (86.1-100)
CCNA1	24	104	0 (0-11.7)	100 (97.2-100)
DCC	27	135	0 (0-10.5)	100 (97.8-100)
MINT31	28	42	0 (0-10.2)	100 (93.1-100)
p16	39	102	0 (0-7.4)	100 (97.1-100)
RARB	13	85	0 (0-20.6)	100 (96.5-100)

B. Best combination of genes for hypermethylation HNSCC detection on serum

Panel	Case (*n*)	Control (*n*)	Sensitivity, % (95% CI)	Specificity, % (95% CI)
CCND2 + TIMP3 + HIC1 + PGP9.5	40	182	65.0 (48.3-79.4)	72.0 (64.9-78.4)
TIMP3 + HIC1	52	278	50.0 (35.8-64.2)	84.5 (79.7-88.6)
CCND2 + HIC1 + PGP9.5	42	189	52.4 (36.4-68.0)	81.0 (74.6-86.3)
CCND2 + HIC1	49	248	44.9 (30.7-59.8)	87.1 (82.3-91.0)
TIMP3 + HIC1 + PGP9.5	45	183	57.8 (42.2-72.3)	74.3 (67.4-80.5)
CCND2 + TIMP3 + HIC1	46	178	56.5 (41.1-71.1)	73.6 (66.5-79.9)
CCND2 + TGFBR2 + TIMP3 + HIC1 + PGP9.5	36	130	72.2 (54.8-85.8)	56.9 (48.0-65.6)
CDH1 + CCND2 + TIMP3 + HIC1 + PGP9.5	39	208	87.2 (72.6-95.7)	42.3 (35.5-49.3)
TGFBR2 + TIMP3 + HIC1	43	158	60.5 (44.4-75.0)	68.4 (60.5-75.5)
TGFBR2 + TIMP3 + HIC1 + PGP9.5	38	131	68.4 (51.4-82.5)	58.8 (49.9-67.3)
TGFBR2 + HIC1	44	149	50.0 (34.6-65.4)	76.5 (68.9-83.1)
CDH1 + TIMP3 + HIC1	49	267	69.4 (54.6-81.8)	57.3 (51.1-63.3)
CDH1 + CCND2 + HIC1 + PGP9.5	39	217	76.9 (60.7-88.9)	49.3 (42.5-56.2)
HIC1	70	373	31.4 (20.9-43.6)	92.5 (89.3-95.0)
TGFBR2 + HIC1 + PGP9.5	39	118	56.4 (39.6-72.2)	66.9 (57.7-75.3)
CCND2 + TGFBR2 + TIMP3 + HIC1	40	126	65.0 (48.3-79.4)	58.7 (49.6-67.4)
CCND2 + TGFBR2 + HIC1 + PGP9.5	37	117	59.5 (42.1-75.3)	65.0 (55.6-73.6)
CDH1 + TIMP3 + HIC1 + PGP9.5	42	209	81.0 (65.9-91.4)	43.5 (36.7-50.6)
HIC1 + PGP9.5	57	202	38.6 (26.0-52.4)	84.2 (78.4-88.9)

promoters previously described as methylated in HNSCC as well other solid tumors. However, we also found that normal control tissue showed substantial rates of methylation in a subset of these promoters. This would suggest that prior studies simply missed the phenomenon of promoter methylation in tissues from subjects without a cancer diagnosis due to small sample size. In addition, this observation could be explained by the phenomenon of compartment-specific methylation as a normal physiologic state. For example, RARB is hypermethylated in normal control salivary rinses at a similar frequency and magnitude when compared with primary HNSCC. Similar phenomena have been noted with other genes (40). Finally, promoter hypermethylation can be associated with age, race, or tobacco and alcohol exposure (27, 42, 43).

These effects may be combined in that studies showing very high specificity for hypermethylated genes in solid tumors often will use a few controls that may be biased to include young, nonsmoker, nondrinker controls, contributing toward a selection bias that artificially increases the false-negative frequency of promoter hypermethylation in controls. Often studies do not include control samples but only determine frequency of promoter hypermethylation in HNSCC primary tumor, salivary rinses, or serum from patients (34). It is important to notice that the control population in the current study can be considered as high risk for HNSCC; the majority of them were male, with median age ~60 years and reported regular consumption of tobacco and alcohol. Due to concerns about age, gender, and tobacco or alcohol consumption as being described as associated to methylation, we did one additional analysis based on salivary rinse samples from cases and controls. The results included the frequency distributions AUC, sensitivity, and specificity for each gene, which was summarized using both continuous and dichotomous methylation status (Supplementary Table S4). On this analysis, we could observe that the genes that were most specific for distinguishing cases and controls did not significantly change the AUC based on age, gender, and tobacco or alcohol consumption (*CCNA1*, *DAPK*, *DCC*, *MGMT*, *TIMP3*, *MINT31*, *p16*, *AIM1*, *ESR*, and *CCND2*). On the other hand, genes such as *HIC1*, *TGFBR2*, *PGP9.5*, *MINT1*, and *CDH1* showed an important variance on AUC results depending on those factors. These results reinforce the observation that genes that are able to discriminate cases from controls in salivary rinse assays were not significantly influenced by age, gender, and tobacco or alcohol consumption. Finally, the use of Q-MSP may have increased our sensitivity in detecting low quantity methylation

even in a subset of healthy controls. However, we noted that the use of Q-MSP as a continuous variable did not show significant improvement compared with analysis as a binary variable.

We did find that the presence of promoter hypermethylation for selected genes proved to be highly specific for HNSCC in primary tumors (e.g., *p16* and *MINT31*; Table 1). However, promoter hypermethylation is not always detectable in salivary rinses or in the serum from HNSCC patients despite the presence of methylation in primary tumors (Table 2A), confirming other reports (7, 12, 26, 30). This may be due to dilution effect of normal, nonmethylated genomes present in salivary rinses from normal mucosa.

We also noted that there was significant variation in the shedding of tumor-specific methylated DNA into the serum compartment. For example, hypermethylated DNA was often not detected in the serum despite the fact that primary tumors were hypermethylated for that specific target. One possible explanation for this is that we were able to detect very low levels of methylation in a small minority of tumors and that this would reduce the net amount of methylated DNA found in serum (e.g., *DCC*, *CCNA1*, and *MINT31*). On the other hand, some genes were found to be methylated in normal mucosa at low levels but would show a higher quantity of methylation in primary HNSCC and were useful as markers for detection in circulating serum. For example, the use of Q-MSP

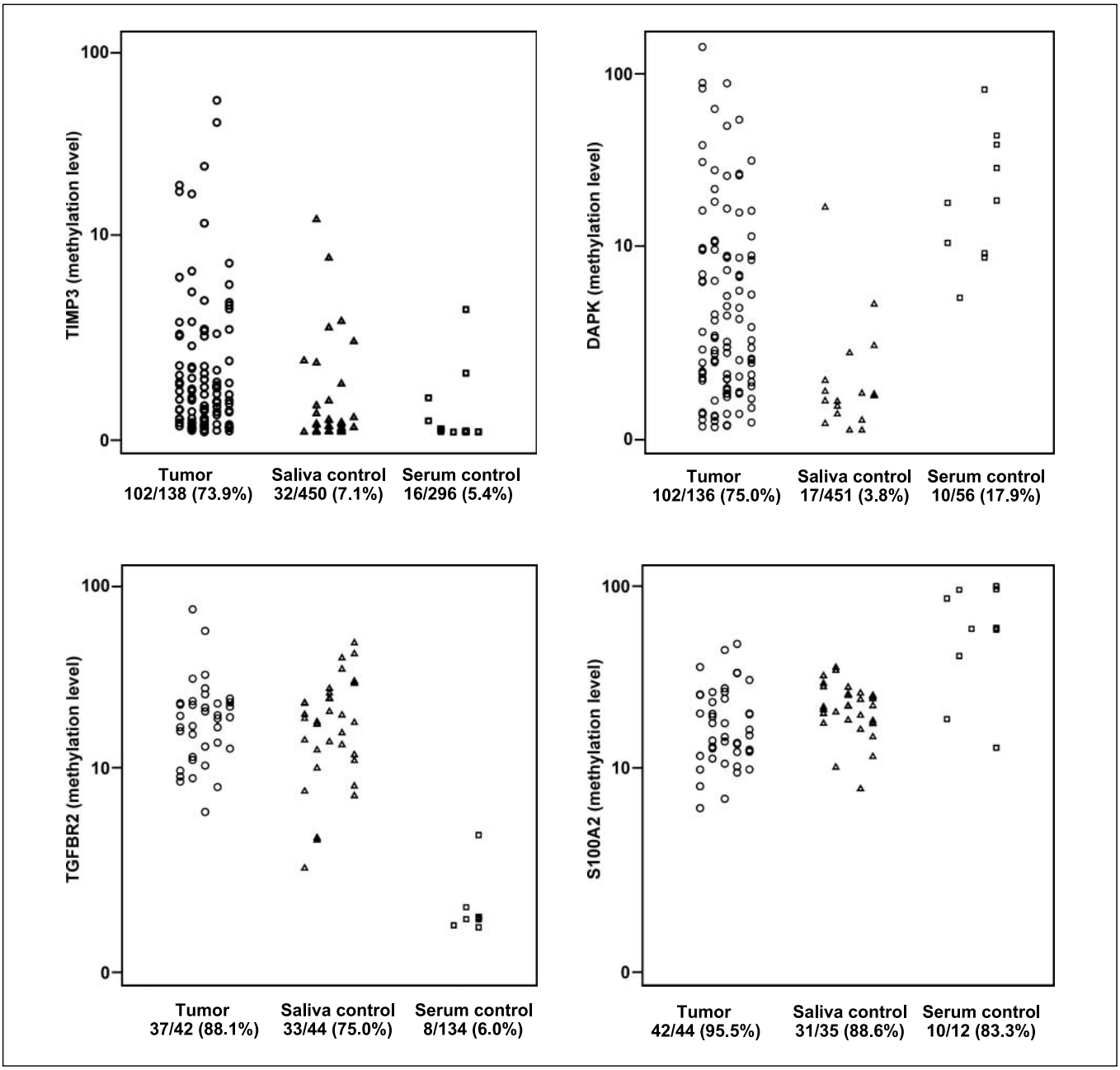

Fig. 3. Compartment-specific methylation considering methylation patterns on tumor, saliva from controls, and serum from controls for selected genes. *X* axis, proportion of methylated cases/tested cases for each sample type; *Y* axis, quantity of hypermethylation (gene of interest/ACTB × 100).

allowed discrimination between elevated levels of promoter methylation in serum when comparing HNSCC patients with normal controls despite similar rates of promoter methylation (*HIC1* and *PGP9.5*). Finally, lack of detection in serum may be due to differential shedding of tumor DNA into the serum compartment that is tumor specific, also noted in other studies.

We were able to show that whereas the use of single genes for detection is possible, using a combination of genes in a panel provides improvement in sensitivity. As promoter hypermethylation patterns in individual tumors show variation depending on specific altered molecular pathways, the use of multiple genes will provide greater applicability and coverage for diverse tumors when compared with a single gene for general detection. From the initial screening of 21 genes for salivary rinses, ultimately 8 genes were selected as part of a panel to distinguish salivary rinses from HNSCC patients and healthy controls (Table 2B). A combination of three or four genes is able to provide a sensitivity ranging from 24.0% to 35.1% with a specificity ranging from 90.0% to 97.1%.

From the six selected genes based on preliminary analysis for use in detection in serum, only *HIC1* would be useful as a single gene marker, with a sensitivity of 31.4% and a specificity of 92.5%. Multiple gene combinations would add a much higher sensitivity (>65%) but would also be associated with a much lower specificity (<60%). However, other gene combinations could have sensitivity of 50.0% with a specificity of 84.5% (Table 4B). Prior reports using promoter hypermethylation to detect HNSCC in serum have shown an ~35% correlation of primary tumor methylation with matched serum for most studies (12, 26, 30, 44). In our study, the overall sensitivity in detecting the HNSCC in serum ranged from 31.4% to 87.2%, with the necessary caveat that improved sensitivity was obtained at the cost of decreased specificity. The relatively low sensitivity of serum-based detection using promoter hypermethylation has been described for other solid tumors as well (45).

In general, this study indicates that adequate assessment of the utility of promoter hypermethylation in HNSCC includes quantitative measurement of promoter methylation as well as a significant sized cohort of appropriately matched normal controls (46). In addition, the presence of promoter hypermethylation of tumor suppressor genes in control populations can happen as a random and perhaps even physiologic event. This methylation may be tissue specific and may also be related to age or environmental carcinogenic exposures (40, 42). These factors significantly affect the selection of a control group, as limited size, young, healthy control group with minimal tobacco and ethanol exposure can bias reporting of falsely elevated specificity for candidate genes.

We did not investigate the complete promoter region, so the functional status of the gene (silencing of the gene) is not necessarily associated with our detection of methylation. However, the aim of this study was to evaluate performance as a biomarker rather that an indicator of functional alteration in primary tumor.

One limitation of this study was the fact that we were unable to have all genes evaluated in all samples (justified by priority of sample usage). Consequently, we may have missed minor associations between methylation markers or risk factors or have been unable to provide a more complete description of methylation in specific compartments. However, there were adequate evaluable samples for the various marker panels analyzed. For the top 21 combinations of markers to detect HNSCC in salivary rinses, 3 of the combinations that had the lowest number of cases available for analysis included 150 cases and 240 controls. The other top 18 combinations had at least 174 cases and 416 controls (Table 2B). For the serum panel, the lowest number of cases available for one of the top marker panels was 36 cases and 130 controls, but other combinations included 70 cases and 373 controls (Table 4B).

In general, we were able to define a panel for HNSCC detection with a high specificity but accompanied by a low sensitivity. This combination of characteristics would not be advantageous for population-based screening. However, we were able to define panels with high sensitivity and low specificity, which may have potential use for surveillance after a HNSCC treatment or surveillance in a high-risk population. Recent studies using the technology of CpG island microarray may be of use in helping to create a panel with higher sensitivity keeping the high specificity (47, 48).

Our findings have also shown that addition of novel detection markers in this context will focus on markers with high specificity, as the effect of addition of markers to a preexisting panel can only degrade specificity. Fortunately, there are some markers (p16 and DCC) that exhibit fairly high specificity, and additional markers with similar, highly specific characteristics are likely to be discovered or characterized in future studies.

Finally, this is an exploratory study in a single cohort of patients. As with all screening and detection modalities, an optimum combination of genes for hypermethylation-based HNSCC detection on salivary rinses or serum needs to be further validated in an independent cohort.

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