Soluble CD44 Is a Potential Marker for the Early Detection of Head and Neck Cancer

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Abstract

Introduction: Head and neck squamous cell carcinoma (HNSCC) is a devastating and deadly disease, largely because it is diagnosed in late stage. Cure rates, currently at 50%, could increase to >80% with early detection. In this study, we evaluate soluble CD44 (solCD44) as an early detection tool for HNSCC by determining whether it reliably distinguishes HNSCC from benign disease of the upper aerodigestive tract.
Methods: We carried out the solCD44 ELISA on oral rinses from 102 patients with HNSCC and 69 control patients with benign diseases of upper aerodigestive tract to determine the sensitivity and specificity of the test for differentiating HNSCC from benign disease. Furthermore, we did a pilot study using methylation-specific PCR primers on oral rinses from 11 HNSCC patients with low solCD44 levels and 10 benign disease controls.

Results: Mean salivary solCD44 levels were 24.4 ± 32.0 ng/mL for HNSCC patients (range, 0.99-201 ng/mL) and 9.9 ± 16.1 ng/mL (range, 0.73-124 ng/mL) for the patients with benign disease ($P < 0.0001$). Depending on cutoff point and HNSCC site, sensitivity ranged from 62% to 70% and specificity ranged from 75% to 88%. Nine of 11 HNSCC and 0 of 10 controls with low solCD44 levels showed hypermethylation of the *CD44* promoter.
Conclusions: SolCD44 is elevated in the majority of HNSCC and distinguishes cancer from benign disease with high specificity. Whereas the solCD44 test lacks sensitivity by itself, methylation status of the *CD44* gene seems to complement the solCD44 test. Our pilot data indicate that, together, these markers will detect HNSCC with very high sensitivity and specificity. (Cancer Epidemiol Biomarkers Prev 2007;16(7):1348–55)

Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for most cancers of the mouth, pharynx, and larynx (1). Each year, 40,000 people in the United States (2) and 500,000 people worldwide (3) are diagnosed with the disease. Tobacco and alcohol exposure are the main risk factors and account for ~85% of HNSCC (4). Only 50% of patients are cured with initial therapy (5). Due to the complex anatomy of the head and neck and equally complex treatment regimens, patients who are cured often suffer from debilitating speech, swallowing, and breathing problems. The majority of HNSCC is identified in advanced stage (6). Early disease (stage I and II) is less debilitating and survival rates reach 80% compared with <40% for late stage disease (stage III and IV; refs. 4, 7).

Early detection of HNSCC would improve treatment outcomes (8, 9) and may be cost-effective if directed at known high-risk populations (10). The detection rate of HNSCC increases from 1 in 1,000 asymptomatic individuals >50 years of age to 1 per 200 high-risk smokers and drinkers (7). In addition to tobacco and alcohol exposure, other factors increase susceptibility to HNSCC. According to Surveillance, Epidemiology, and End Results data, black males have a higher incidence of HNSCC, present at later stage, and have

lower 5-year survival and higher mortality than the general population (11). Low socioeconomic status may also be associated with increased risk of HNSCC (12, 13). Considering that minority patients and those of low socioeconomic status suffer disproportionately from this disease, such an early detection test should be noninvasive, easy to administer, and inexpensive so that patients with limited access to expert diagnosis might also have access to screening.

Most HNSCC screening trials to date use physical exam as the screening method. Physical exam–based screening is expensive (10), has limited sensitivity and specificity (14-16), and cannot detect occult disease (17). Furthermore, despite the emphasis of American Cancer Society and National Cancer Institute that oral examinations could prevent many deaths, few individuals receive these exams (18).

Because screening by physical exam has these drawbacks, research is focusing on molecular solutions. Much of the work on HNSCC tumor markers done in serum has been replicated in whole unstimulated saliva or oral rinses. These media have the advantage over blood in that they are readily accessible without needle stick.

Molecular early detection tools can be divided into nucleic acid–based and protein-based markers. Hu et al. (19) reviewed many of the nucleic acid–based markers, which include loss of heterozygosity, microsatellite instability, p53 mutations, abnormal promoter hypermethylation, and mitochondrial DNA mutations. Telomerase activity has also been studied using a PCR-based assay (20). More recently, Wong's group, using quantitative PCR analysis of mRNA in saliva, showed that a panel of four markers detect HNSCC with 91% sensitivity and 91% specificity (21). Such feasibility studies show promise, but none of these markers have been validated in large trials. Furthermore, most of these methods do not fulfill the ideal

characteristics of a tumor marker because they are relatively expensive to carry out, require considerable expertise, and are not widely available.

Unlike nucleic acid–based techniques, protein-based early detection tools detect posttranscriptional and posttranslational changes that may take place as a result of tumorigenesis. Surface-enhanced laser desorption/ionization-time of flight mass spectrometry and antibody microarrays have been used to analyze samples from HNSCC patients and normals to develop a protein level signature for HNSCC (22, 23). However, these techniques are also complex and expensive and therefore not feasible for mass screenings. The ELISA system is cited as the most sensitive, well-established, and widely available protein-based testing platform for the detection of cancer in body fluids or tissue (24). Several markers have been studied using ELISA or ELISA-like assays. Some have shown feasibility but have not been validated in large trials. Salivary hyaluronic acid was elevated in 8 of 11 HNSCC patients and 0 of 6 normal controls. Salivary hyaluronidase was elevated in 11 of 11 HNSCC patients and 0 of 6 controls (25). Interleukin-8 detected HNSCC in saliva with 86% sensitivity and 97% specificity in 32 HNSCC and matched controls (26). SolCD44 was elevated in oral rinses from 19 of 24 HNSCC patients with disease invading mucosa and none of 10 normal controls (27).

Whereas each of these markers shows potential, we are particularly interested in CD44 because it seems to be a relatively early marker of malignancy. In normal upper aerodigestive tract epithelium, CD44 is expressed at the basal surface. However, in epithelium with histologic dysplastic changes, CD44 expression increases to involve all layers of the epithelium (28, 29) in 90% of cases. This overexpression is seen not only in dysplasias but also in 90% of invasive HNSCC (29). CD44 comprises a family of isoforms that arise from alternative splicing of a region of variable exons (exons 5-14; ref. 30). They differ in primary amino acid sequence as well as in amount of N- and O-glycosylation (31). A few isoforms such as CD44 standard (CD44s) and CD44v3-10 are found in normal cells, whereas CD44 variant isoforms (CD44v) are differentially expressed in some tumors and promote tumorigenesis (32).

Overexpression of normal isoforms occurs in many tumor tissues and also promotes oncogenesis (32). CD44 undergoes interactions with other molecules such as members of the ErbB and matrix metalloproteinase (MMP) families (33). One member of the MMP family, membrane type 1 MMP, cleaves CD44 to its soluble form (solCD44), resulting in increased cell migration (34, 35). SolCD44 levels correlate with metastases in some tumors (36, 37). Studies in HNSCC tissues show increased membrane type 1 MMP expression in tumors with lymph node metastases (38), which could contribute to increased solCD44 in bodily fluids such as saliva.

Studies involving solCD44 in HNSCC have been few and were reviewed in our pilot study (27). Briefly, for HNSCC, plasma levels of a solCD44 isoform were not significantly elevated in HNSCC patients versus controls. This finding was attributed to epithelial compartments such as breast and colon, which also release the isoform into the circulation creating a basal plasma level. Because the tumor load of HNSCC is relatively low, solCD44 released from these tumors is not able to significantly exceed the basal level (39). A recent report shows that mean solCD44 levels in serum are almost 2.5 times higher in HNSCC patients compared with normal controls (40). In oral rinses, our pilot study indicated that solCD44 levels were >7 times higher in HNSCC patients compared with normal controls. Furthermore, levels were elevated in almost 80% of the HNSCC patients and none of the controls (27). Therefore, whereas CD44 seems to be a promising tumor marker, it may be more effective as a detection tool when samples are collected from the local environment as an oral rinse than from the circulation. However, benign conditions such pharyngitis, sinusitis, and reflux may also increase solCD44 levels in oral rinses. To be useful as an early detection test, the solCD44 test will have to be able to distinguish HNSCC from benign diseases of the upper aerodigestive tract.

In our pilot study, >20% of patients with HNSCC had low solCD44 levels (27). There is evidence that both overexpression and underexpression of CD44 are associated with poor prognosis (41, 42). It is possible that in some HNSCC patients, the CD44 gene is turned off by promoter hypermethylation. DNA hypermethylation plays an important role in carcinogenesis (43). Hypermethylation occurs by enzymatic addition of a methyl group to the carbon-5 position of cytosine. The majority of methylated cytosines occur as 5'-CpG-3' dinucleotides that are distributed in islands (44) associated with housekeeping genes and genes with tissue-specific patterns of expression (43). Normally, these islands are unmethylated. When hypermethylated, gene suppression occurs. Many genes, including those involved with tumor suppression, cell adhesion, and hormone response, are known to undergo hypermethylation that is associated with tumor progression (43). Work in fibrosarcoma animal models and other cancers such as prostate suggests that in some instances, the CD44 gene is turned off by promoter hypermethylation (45, 46). To our knowledge, no studies have been done to determine if CD44 expression is turned off by promoter hypermethylation in HNSCC.

In this report, we evaluate the solCD44 ELISA test for HNSCC in a larger group of patients. We examine the test in its ability to distinguish between patients with benign conditions and those with malignant disease, assess the effect of demographics and risk factors on results, and present our preliminary data on solCD44 levels in dysplasia patients. We suspect that HNSCC patients but not controls with low solCD44 levels have undergone gene silencing by promoter hypermethylation. If this is the case, methylation status of the CD44 gene and the solCD44 test may complement each other and, in combination, distinguish HNSCC with high sensitivity and specificity. We will test this hypothesis in a pilot study of 11 HNSCC patients and 10 controls with low solCD44 levels.

Materials and Methods

Subject Characteristics. One hundred two HNSCC patients and 69 controls were enrolled from otolaryngology clinics at the University of Miami Sylvester Comprehensive Cancer Center and Jackson Memorial Hospital. Our pilot data showed that solCD44 levels in oral rinses were significantly elevated compared with normal volunteers. We hypothesized that levels may be elevated in benign reactive, hyperplastic, and inflammatory processes in addition to invasive HNSCC. These conditions are common and likely to be prevalent in the proposed high-risk screening population. If solCD44 levels are substantially elevated in benign disease, the test will not likely be useful for the diagnosis of cancer. To evaluate this, our control group consisted entirely of patients seeking treatment for benign disease of the upper aerodigestive tract (hereafter referred to as benign disease). To ensure that benign disease patients included mainly smokers and drinkers (as is true of the HNSCC population), they were approached if they answered ''yes'' to tobacco or alcohol use on the clinic intake questionnaire. Control patients were excluded if they had a potentially malignant lesion or if final diagnosis of their condition was unknown. One control patient was excluded when a severely dysplastic lesion was diagnosed in follow-up. This patient was placed in a group of six dysplasia patients who were studied separately. All HNSCC patients had biopsy-proven, newly diagnosed or recurrent HNSCC. We included all stages and sites except nasopharynx because nasopharyngeal carcinoma tends to behave differently than HNSCC in

other sites. Subjects known to be pregnant or infected with HIV were excluded. All subjects were enrolled according to the protocol approved by the Institutional Review Board and completed a written consent before enrollment.

Saliva Collection. Five milliliters of normal saline were placed in the subjects' mouths. Patients were asked to swish for 5 s, gargle for 5 s, and then spit into a specimen cup. The resulting oral rinse was placed on ice for transport and stored at −80°C.

SolCD44 ELISA. We chose to measure levels of solCD44s using an ELISA assay (Bender MedSystems) that recognizes all CD44 normal and variant isoforms. This assay has been extensively used in serum and other body fluids (35, 39) and correlates with cancer progression in many tumors (35, 36). Specificity of the CD44 antibody is described in detail in the Bender MedSystems Manual. They detected no cross-reactivity between this test and tumor necrosis factor α, tumor necrosis factor β, tumor necrosis factor receptor, IFN-α2c, IFN-γ, interleukin-8, annexin, soluble endothelial leukocyte adhesion molecule 1, soluble L-selectin, soluble intercellular adhesion molecule 1, or human epidermal growth factor receptor 2. We have also previously confirmed specificity of the antibody by Western blot (27).

The principles of the test involve a sandwich-type ELISA in which a monoclonal anti-solCD44 antibody, adsorbed onto microwells, binds CD44 in the sample. Horseradish peroxidase–conjugated monoclonal anti-solCD44 antibody binds the CD44-antibody complex and reacts with a substrate solution to produce a colored product with an absorbance measured quantitatively at 450 nm. Sample concentrations are determined by a standard curve. We have modified the test since our original pilot report. The Bender MedSystems ELISA plate is designed for use with plasma, serum, and urine samples. Any matrix (i.e., serum, urine, or saliva) may contain factors that affect ELISA test results. This is known as a matrix effect. Such effects can be corrected by running the standards in the same matrix as the samples. To better adapt the test to saliva specimens, we now prepare our standards in a synthetic saliva matrix (Salimetrics) diluted 1:5 in normal saline (because patients swish and gargle with 5-mL saline) and switched to a sample diluent (Salimetrics) developed for saliva samples. As previously reported, samples were vortexed, centrifuged at 3,000 × g, and the supernatant was used for study. In many cases, the pellet was saved for future studies. The Bender MedSystems recommended dilution of 1:60 resulted in non-detectable levels of solCD44. Instead, we did the test as directed at full concentration and 1:2 and 1:4 dilutions. In an effort to correct for patients' varying hydration status, we normalized the solCD44 levels to protein as done by Lokeshwar's group for the salivary hyaluronic acid-hyaluronidase test (24). We did the protein assay (Bio-Rad) according to the manufacturer's protocol using saliva samples at three dilutions. All sample assays were done in triplicates. The protein and solCD44 concentrations for each sample were averaged and divided by the average protein concentration for that sample.

SolCD44 ELISA Quality Control. The standard curve was generated using cubic spline curve fit. Standard curves were run in duplicate on each plate. The curve was linear with an *r* value of 0.98 to 0.99.

The precision of an assay is defined by the agreement between replicate measures (47). Samples (73 HNSCC and 54 control specimens) were repeated in triplicates at full concentration and 1:2 and 1:4 dilutions. The average coefficient of variation for the resulting 381 duplicate measurements was 4.5%.

Analytic sensitivity is defined as the lowest concentration detected that is significantly different than zero (47). Mean absorbance of our blanks run on 17 different plates was 0.02 ± 0.015. We defined 3 SD as significantly different and calculated the corresponding concentration from a representative standard curve. Using this method, the analytic sensitivity of the test is 0.091 ng/mL.

Samples were run on a total of 17 ELISA plates. Because a reference standard is not available, we prepared the positive control sample containing 59 ng/mL recombinant solCD44 in synthetic saliva diluted 1:5 in normal saline. This positive control was run in triplicates on each plate to assess differences between plates. Average coefficient of variation for triplicates readings of the positive control was 3.6%. Coefficient of variation between plates was 9.7%.

Statistical Analysis. Statistical analyses were done using programs of the SAS Institute, Inc. (version 9.1). The mean solCD44 levels and protein levels were calculated separately for HNSCC patients and patients with benign disease with corresponding confidence limits. Resulting means for HNSCC patients were compared with means for patients with benign disease using the Student's *t* test. We also compared solCD44 and protein levels between specific subgroups of cancer patients based on characteristics such as stage, site, and tumor size. The five patients without disease at the primary site were excluded from this analysis. Student's *t* test was used in most cases because only two groups were compared. ANOVA was used in cases where more than two groups were compared.

A good screening test for HNSCC must have high sensitivity and specificity. Using results from 102 HNSCC patients and 69 subjects without HNSCC, the sensitivity and specificity of the solCD44 test were calculated at several cutoff points, thereby deriving its receiver-operator characteristic curve.

Because HNSCC is tightly linked to risk factors such as tobacco and alcohol, we made efforts to control for such factors. Patients most recently accrued and completed a questionnaire containing information on potentially important covariates, including tobacco and alcohol exposure, race, ethnicity, gender, and socioeconomic status. In addition, they received a head and neck examination. We have this information available on 43 stage I-IV newly diagnosed HNSCC and 63 controls with benign disease. We compared the two groups using Student's *t* test. The distribution of potentially important covariates was compared between the two groups by χ^2 analysis.

***CD44* Hypermethylation.** Whereas CD44 overexpression seems to promote HNSCC tumorigenesis, some HNSCC patients showed low solCD44 levels in their oral rinses, indicating that the *CD44* gene may be turned off in some cancer patients. Because hypermethylation is known to turn off the *CD44* gene in other cancers, we did methylation-specific PCR to determine if low solCD44 levels may be due to *CD44* promoter hypermethylation. Subjects included 11 HNSCC and 10 benign disease control subjects enrolled in the main solCD44 study who had low solCD44 levels and adequate DNA in their oral rinses. Controls were selected so that the group was similar to the HNSCC patients with respect to tobacco and alcohol exposure, age, race, gender, and solCD44 level. For this study, we used the oral rinse pellets, collected as described above. DNA was isolated using a QIAmp DNA mini kit (Qiagen) as per manufacturer's protocol. We previously used described methods of Singal et al. (48). Methylation-specific PCR was done using primer sequences specific for the modified DNA, but not wild-type DNA, designed by Dr. Singal's group (forward, *CD44* MS-5′-AGTTTTAGTAGA-GTACGGGGC-3′; primer, *CD44* MS-5′-ACGAACGAAAAA-CACACCCAAACA-3′). An additional PCR reaction using primers specific for β-actin was used as a control to verify that DNA was present in the samples (48). The experiment was repeated with specimens from HNSCC cases and controls tested in the same PCR run.
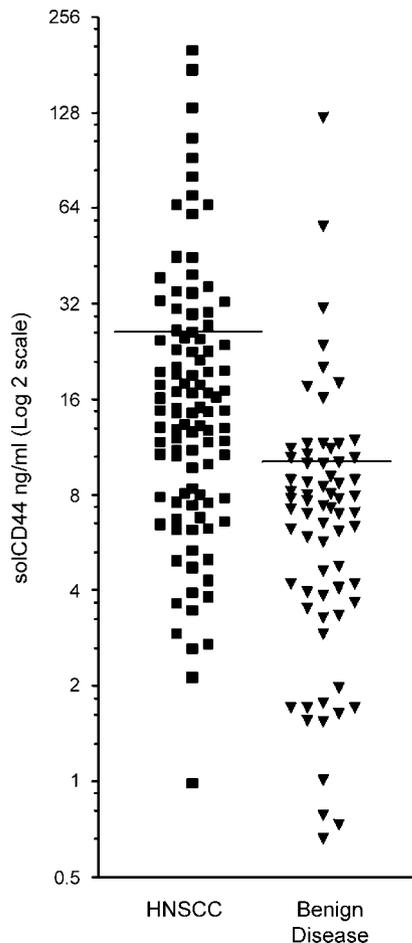
Figure 1. Salivary solCD44 levels are elevated in HNSCC patients compared with normal nonsmokers and patients with benign disease. *Y* axis was transformed to log 2 format to aid visualization of differences. *Horizontal lines,* means of both groups. Differences between HNSCC patients and the two groups without cancer both were highly statistically significant (*P* < 0.0001).

Results

SolCD44 Levels. The mean solCD44 level was 24.4 ± 32.0 ng/mL for HNSCC patients and 9.9 ± 16.1 ng/mL for the patients with benign disease (*P* < 0.0001). Results are shown in Fig. 1. Because SDs were large, we did a diagnostic procedure using SAS software to identify outliers. The software identified seven samples that met outlier criteria. They all had solCD44 levels >80 ng/mL. Six were from tumor patients and one was a benign disease patient. When these were removed from the analysis, the SD decreased to less than the mean value for all tumor characteristics, and differences in means between HNSCC and benign disease were still highly statistically significant (HNSCC: mean, 17.76 ± 14.23; benign disease: mean, 8.25 ± 8.13; *P* < 0.0001). Because the solCD44 test is still under investigation, we chose to continue the analysis including the outliers.

Interestingly, five HNSCC patients with cervical lymph node squamous cell carcinoma but no identified upper aerodigestive tract primary lesion had elevated mean solCD44 levels compared with the combined control group (19.2 ng/mL; *P* = 0.15, *t* test; *P* < 0.01, nonparametric test). Whereas none of them had a visible malignant lesion in the upper aerodigestive tract by endoscopy with appropriate biopsies, three had previous HNSCC and the other two had unknown primaries, where the origin of the squamous cell carcinoma was not

identified. The unknown primary HNSCC is a well-described entity and occurs in ~5% of cases (13). Elevated solCD44 levels were present in one of two unknown primary patients and suggest that the solCD44 test can detect occult invasive disease. The high levels in all three patients with a history of HNSCC and recurrence in the neck raise the possibility that the solCD44 test detects HNSCC malignancy in its earliest stages before invasion occurs.

SolCD44 levels were higher in patients with oral cavity and oropharynx tumors compared with patients with larynx and hypopharynx tumors (*P* < 0.01). Levels did not correlate significantly with tumor stage, tumor size, presence of lymph nodes, history of previous HNSCC (recurrence or second primary), or history of prior radiation (Table 1).

Whereas the HNSCC patients and controls did not vary significantly with respect to race or ability to gargle, by χ^2 analysis, cancer patients were significantly older, more likely male, less educated, reported less income, had poorer dentition, used more tobacco products, and drank more alcohol (Table 2). As shown in Table 3, the unadjusted effect for cancer compared with controls is, on average, 10.32 units higher for solCD44. Bivariate adjustments for important characteristics in Table 2 did not diminish the effect of group on the level of solCD44. After bivariate adjustment, cancers were, on average, 8.32 units higher than controls for teeth removed (*P* = 0.053) to as much as 13.5 units higher than controls for income level (*P* = 0.0015). Therefore, although the groups were not balanced with respect to some known risk factors, the adjusted analysis provides strong indication that there is indeed higher expression of solCD44 in HNSCC cancer patients compared with subjects with benign disease.

Patients with HNSCC are at risk of developing a second primary in the upper aerodigestive tract because of the combination of a field effect and clonal outgrowth associated with upper aerodigestive tract squamous cell carcinogenesis (49, 50). Upper aerodigestive tract mucosa passes through progressive stages of dysplasia over a number of years before invasion (51). Therefore, patients with a history of HNSCC are expected to have multiple areas of dysplasia throughout their upper aerodigestive tract, even after the initial primary tumor is treated. This, combined with immunohistochemical evidence that CD44 is an early marker of malignant change in the upper aerodigestive tract (27, 28) and the fact that dysplasia can be clinically occult (52), led us to suspect that the solCD44 test may be detecting dysplasia in the three patients with

Table 1. Means salivary solCD44 level by tumor characteristic

	N	Mean (SD)	P
Group			
Benign disease	69	9.932 (16.121)	0.0001
Cancer	102	24.444 (32.013)	
Tumor site			
Larynx/hypopharyx	35	15.112 (32.013)	0.0057
Oral cavity/oropharynx	62	30.134 (39.382)	
Tumor stage			
I-II	40	21.384 (25.009)	0.4060
III-IV	62	26.418 (35.873)	
Nodal status			
N_0	65	20.450 (23.849)	0.1510
N_1, N_2, N_3	37	31.460 (42.245)	
Recurrence status			
No recurrence	87	23.636 (32.312)	0.5422
Recurrence	15	29.127 (30.863)	
Second primary			
Second primary	8	28.864 (29.064)	0.6862
No second primary	94	24.068 (32.366)	
Radiation therapy			
No radiation therapy	85	23.003 (31.720)	0.3117
Radiation therapy received	17	31.649 (33.480)	

Table 2. Characteristics of cancers versus normals (n = 106)

	Study group		P
	Benign disease (n = 63)	Cancer (n = 43)	
	n (%)	n (%)	
Age group			
<60 y	51 (80.95)	20 (46.51)	0.0002
>60 y	12 (19.05)	23 (53.49)	
Race			
Non-White	10 (16.13)	3 (7.14)	0.1739
White	52 (83.87)	39 (92.86)	
Gender			
Female	31 (49.21)	8 (18.60)	0.0013
Male	32 (50.79)	35 (81.40)	
Education level			
More than high school	54 (85.71)	17 (39.53)	<0.0001
No school-high school	9 (14.29)	26 (60.47)	
Income level			
<$35K	7 (12.07)	24 (64.86)	<0.0001
>$35K	51 (87.93)	13 (35.14)	
Ever smoked cigarettes			
Insufficient data	0 (0.00)	1 (2.33)	0.0026
No	26 (41.27)	5 (11.63)	
Yes	37 (58.73)	37 (86.05)	
Current cigarette use status			
Current	13 (20.63)	20 (47.62)	0.0013
Former	24 (38.10)	17 (40.48)	
Never	26 (41.27)	5 (11.90)	
Ever used any tobacco product			
At least 1 tobacco product	45 (71.43)	42 (97.67)	0.0005
Never used any tobacco	18 (28.57)	1 (2.33)	
Drinking level			
Heavy drinker	3 (4.92)	7 (17.50)	0.0003
Light drinker	34 (55.74)	8 (20.00)	
Moderate drinker	15 (24.59)	8 (20.00)	
Nondrinker	9 (14.75)	17 (42.50)	
Gargle (3 levels)			
Fair	10 (16.39)	10 (24.39)	0.3546
Good	50 (81.97)	29 (70.73)	
Poor or none	1 (1.64)	2 (4.88)	
Teeth removed			
1-5	19 (30.16)	16 (37.21)	0.0003
6 or more	8 (12.70)	9 (20.93)	
All	1 (1.59)	9 (20.93)	
None	35 (55.56)	9 (20.93)	

recurrence in the neck but no mucosal primary. To further investigate this, we analyzed solCD44 levels in patients with dysplasia but without any invasive disease. Details of presentation and treatment are provided in Table 4.

Occult dysplasia is probably the explanation for elevated solCD44 levels in patient 6. Whereas no lesions were seen at the time of collection, the patient developed a clinically evident lesion and biopsy-proven severe dysplasia over 2 years later. Subject 2 also fits this scenario. This patient had a very high solCD44 level, a worrisome lesion on exam, but only a dysplasia on final pathology. Interestingly, the patient has a history of HNSCC and carcinoma in situ (CIS), suggesting that this patient's mucosa is laden with many areas of dysplasia. Subject 5 had a history of CIS of the vocal cord that was removed. An oral rinse was collected shortly thereafter. Whereas a repeat biopsy of the vocal cord was negative for malignancy, the solCD44 level was high. Less than 2 years later, the patient developed two simultaneous, biopsy-proven invasive cancers in the larynx and hypopharynx.

Cutoff Point, Sensitivity, and Specificity. Using results from the complete group of 102 HNSCC patients and 69 benign disease controls, the sensitivity and specificity of the solCD44 test were calculated at several cutoff points, thereby deriving its receiver-operator characteristic curve. A cutoff

point set at 12 ng/mL resulted in a sensitivity of 62% and specificity of 88%. A cutoff point at 10.5 ng/mL resulted in sensitivity of 70% and specificity of 75%. A receiver-operator characteristic curve was created using data from oral cavity and oropharyngeal patients because solCD44 levels were significantly higher in this group. For this group of cancers, a cutoff point at 12 ng/mL resulted in sensitivity of 63% and specificity of 88%. Results are comparable to other widely used screening tests such as prostate-specific antigen for prostate cancer (sensitivity, 60-80%; specificity, 90%; ref. 53) and the Papanicolaou test for cervical cancer (sensitivity, 30-87%; specificity, 86-100%; ref. 54).

With the cutoff point set at 12 ng/mL, there were 39 false-negative results. The solCD44 test correctly detected 70% of T_1, 50% of T_2, 63% of T_3, and 61% of T_4 disease. By stage, the test correctly detected 62% of stage I, 53% of stage II, 67% of stage III, and 63% of stage IV disease. The test also identified 73% of recurrences, 62% of second primaries, and 60% of newly diagnosed HNSCC. The solCD44 test detected 63% of oral cavity, 56% of laryngeal, 63% of oropharyngeal, and 62% of hypopharyngeal primaries.

We investigated whether specific benign diseases of the upper respiratory tract were associated with high solCD44 levels. There were a total of 80 active diagnoses among 63 benign disease patients who completed questionnaires. SolCD44 levels in patients with a specific diagnosis were compared with the remaining control patients using a t test. Specific diagnoses included rhinitis/sinusitis (n = 30), obstructive sleep apnea (n = 7), reflux (n = 11), other diseases of the upper aerodigestive tract (n = 21), and no active disease. The subgroup labeled "other disease" (n = 21) included four patients with tonsillitis or pharyngitis with mean solCD44 level of 6.85 ng/mL. The obstructive sleep apnea group had significantly decreased levels (mean, 4.303; P = 0.0125). No diagnosis was associated with increased levels. When we compared solCD44 levels in a group of 15 normal volunteers (mean, 6.5 ng/mL) to this group of 63 patients with benign disease (mean, 10.17 ng/mL), differences did not reach statistical significance by parametric (P = 0.16) or nonparametric (P = 0.22) test.

With the cutoff point set at 12 ng/mL, there were eight false-positive results. We have information on active upper aerodigestive tract benign disease for eight of the controls with positive results. Three had reflux, two had rhinitis/sinusitis, one had two diagnoses rhinitis/sinusitis and adenoid hypertrophy, one had dysphagia, and one had chronic laryngitis secondary to caustic ingestion. Whereas reflux was not associated with a statistically significant elevation in solCD44 level, 3 of 11 patients with reflux had false-positive results. Reflux is a known risk factor for HNSCC and these patients may be at risk for developing malignant change. Interestingly, the subject who converted to severe dysplasia had no other risk factors for HNSCC other than a history of reflux.

Table 3. Tabulate bivariate adjustment of CD44 levels

	Effect of group	P
Unadjusted	10.32	0.0097
Adjusted for age group	8.99	0.0348
Adjusted for race	9.84	0.0162
Adjusted for gender	11.68	0.0055
Adjusted for education	12.92	0.0046
Adjusted for income	13.45	0.0015
Adjusted for smoked >100 cigarettes	10.18	0.0118
Adjusted for current smoking status	8.47	0.0442
Adjusted for ever used *any* tobacco product	9.08	0.0314
Adjusted for alcohol usage (four groups)	13.06	0.0034
Adjusted for number of teeth removed	8.32	0.0526

Table 4. SolCD44 levels in dysplasia and CIS

#	ng/mL	Site	Presentation at time of collection	Degree of dysplasia	Treatment of dysplasia	Follow-up (mo)
1	4.4	Larynx	Leukoplakia	CIS	Removal	No progression (>48 mo)
2	88.3	Oral cavity	History of HNSCC, new lesion with invasive carcinoma vs CIS on biopsy before collection	Only dysplasia on final pathology after removal	Wide excision	No progression (18 mo)
3	3.0	Larynx	Leukoplakia	CIS	Removal	Dysplastic changes on repeat biopsy, further changes consistent with CIS on 3rd biopsy (17 mo)
4	10.4	Larynx	History of CIS, presents with leukoplakia	Mild-moderate dysplasia	Removal	No progression (14 mo)
5	32.5	Larynx	CIS diagnosed and removed shortly before collection	Repeat biopsy—no dysplasia	Not applicable	Developed leukoplakia of oropharynx 3 mo post collection that resolved, then developed two invasive cancers in the larynx and hypopharynx (24 mo)
6	20.8	Oropharynx	No disease, developed severe dysplasia 2.5 y after collection	Not applicable	Removal	Recurrent mild dysplasia on soft palate

Salivary Protein Levels and Normalized SolCD44. We hypothesized that hydration status may influence solCD44 levels. Because total protein levels correlate with hydration status in whole saliva, we normalized our solCD44 values to total protein to correct for varying hydration status between patients (27). However, mean normalized solCD44 levels between tumors (28.67 ng/mL) and controls (22.54 ng/mL) did not reach statistical significance ($P = 0.27$). On further review, our data showed that protein levels were significantly higher in HNSCC patients (1.08 mg/mL) compared with benign disease patients (0.507 mg/mL; $P < 0.0001$), thus accounting for this loss of significance. We were concerned that protein levels in HNSCC were elevated because the patients were dehydrated because large tumors can cause swallowing difficulties. However, if this were the case, protein levels should increase with increasing tumor size. Our data show no such correlation. Protein levels for T_1-T_4 lesions were 1.0, 0.95, 1.2, and 1.0 mg/mL, respectively. For this reason, we chose not to use normalized values.

CD44 Hypermethylation. Results of methylation-specific PCR on subjects with low solCD44 levels showed that 9 of 11 HNSCC and 0 of 10 normal control subjects had hypermethylated *CD44*. Results are shown in Fig. 2. We verified results by repeating methylation-specific PCR, this time with cases and controls run together. Results were the same in the repeated experiment. Characteristics of the HNSCC cases are given in Table 5. The cases and controls were compared with regard to race, gender, age, ability to gargle, whether they ever smoked, whether they ever used any tobacco, and whether they were nondrinkers/light drinkers versus moderate/heavy drinkers using Fisher's exact test. There were no significant differences between the two groups.

Discussion

A recent report on a large physical exam–based screening program in India supports use of routine oral visual screening to reduce oral cavity mortality (55). However, this method of screening has many limitations and therefore has not become widely accepted. Moreover, oral cavity exam alone will not identify cancers deep in the pharynx or larynx. Our data suggest that the solCD44 test is very specific for HNSCC. It is elevated in the majority of HNSCC and may be useful in the future to identify patients likely to respond to anti-CD44 therapies. However, by itself, the solCD44 test lacks the sensitivity needed to meet criteria for a useful early detection

screening test. Addition of CD44 methylation status or other markers may greatly improve sensitivity without compromising specificity.

Specificity and Subclinical Disease. In our pilot study, we showed that solCD44 levels in oral rinses were >7 times elevated in HNSCC compared with controls. Because CD44 is a ubiquitous protein, we were concerned that solCD44 may be elevated in common benign diseases of upper aerodigestive tract. This could lead to unacceptable false-positive results. This study shows that the solCD44 test is quite specific, distinguishing cancer from benign disease 88% of the time. We are not aware of other studies that have evaluated specificity of a HNSCC marker to this extent.

The specificity of the solCD44 test may be even greater than reported here because there is evidence it detects occult disease. Prospective data on patients with dysplasia (Table 4) suggest that the solCD44 test detects disease before presentation of a visible lesion. One patient who was a nonsmoker and nondrinker had no abnormalities on physical exam. A solCD44 test done at that time was positive. Two and a half years later, she developed a severe dysplasia of the soft palate. Another patient had CIS of the larynx in the past and underwent extensive negative biopsies of the larynx. Despite the negative biopsies, the solCD44 test was positive. Two years later, the patient was diagnosed with biopsy-proven cancers in the hypopharynx and larynx. Because our control subjects included tobacco users and drinkers, some of the false positives may be true positives for occult dysplasia or early invasion.

Before developing frank invasion, upper aerodigestive tract progresses through increasing grades of dysplasia. Dysplasia is a histologic diagnosis and is relatively an innocuous phase
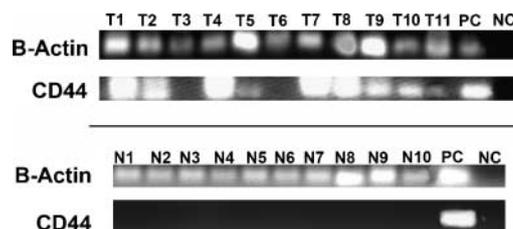

Figure 2. CD44 promoter is hypermethylated in DNA from oral rinses of 9 of 11 HNSCC patients and 0 of 10 benign disease patients with low solCD44. *PC*, positive control; *NC*, negative control.

for the patient. Often, the dysplastic stage is invisible and progresses to malignancy 16% to 36% of the time over a period of 4 to 14 years (51, 52). The true rate of transformation is not known because most severely dysplastic lesions are removed. Dysplasia is easily treatable if the lesion is clinically visible and surgically resectable. A screening test that can detect lesions at this phase would be ideal. Because this phase is also reversible, even if the lesion is clinically occult, a screen-positive individual would have more impetus to stop risky behaviors and start reparative therapy such as improved diet or chemo-preventive measures.

The evidence that solCD44 detects premalignant disease also explains aspects of our data that are otherwise puzzling. For example, all three of the patients with history of HNSCC, recurrence in the neck, and no invasive upper aerodigestive tract disease at the time of collection had elevated levels. According to Slaughter's theory of field cancerization, the entire upper aerodigestive tract comes in contact with carcinogens and thus is at risk for tumorigenesis. This, along with the theory of clonal outgrowth, explains why HNSCC is associated with multiple invasive tumors and why patients with a history of HNSCC have a significant chance of developing a second invasive primary (49, 50). Thus, the three patients with a history of HNSCC and recurrence in the neck at the time of oral rinse collection would be expected to have multiple regions of dysplasia in the upper aerodigestive tract. Because it is known that CD44 expression is increased in dysplastic upper aerodigestive tract mucosa compared with normal mucosa, it is not surprising that solCD44 levels are elevated in this group. Furthermore, it is possible that these patients have occult recurrence at the primary site in addition to clinical disease in the neck.

Sensitivity and Clinically Inaccessible Disease. This study shows that solCD44 is elevated in HNSCC patients compared with benign disease patients and distinguishes cancer from benign disease with 62% sensitivity. The SDs and means between groups were large. Much of this seems to be explained by a few very high solCD44 levels occurring mainly in HNSCC patients. We are including the outliers at this point because the test is still under investigation. Whereas our benign disease controls did not match the cancer patients with respect tobacco and alcohol use, after bivariate adjustment, these risk factors do not seem to affect significance.

Although solCD44 levels are elevated in the majority of HNSCC patients, many have low levels. Mean levels were lower for laryngeal and hypopharyngeal tumors than oral cavity and oropharyngeal tumors. It is generally accepted that a single molecular marker will have inadequate sensitivity and specificity to accurately detect cancer. In the case of solCD44, the main weakness is inadequate sensitivity. A significant proportion of HNSCC patients had low solCD44 levels, which overlapped levels in the benign disease

population. For this reason, we have begun to investigate other markers that, in combination with solCD44, may achieve adequate sensitivity and specificity. We have previously reported our pilot data on hyaluronic acid and hyaluronidase, which are both elevated in saliva of HNSCC patients compared with controls (25) and are good potential constituents of such a marker panel. Here we have examined methylation status of the *CD44* gene both to determine whether it may enhance our marker panel and explain why the solCD44 test lacks sensitivity.

We found that >80% of the HNSCC patients with low solCD44 levels can be distinguished from benign disease controls based on methylation status of the *CD44* gene. To our knowledge, this is the first study showing *CD44* promoter hypermethylation in HNSCC. Immunohistochemistry findings support that both underexpression and overexpression of CD44 are associated with poor prognosis in HNSCC (41, 42). CD44 overexpression and hypermethylation-mediated under-expression have both been verified in a fibrosarcoma mouse model, although they occur at different stages of tumorigenesis (45). Furthermore, the *CD44* gene is hypermethylated in other cancers such as prostate (46).

The specificity of *CD44* methylation status was 100% in this pilot study. This high specificity and ability to detect most of the HNSCC missed by the solCD44 test suggest that the combination of these markers will result in an early detection test with estimated sensitivity and specificity of ~90%. Moreover, two of two laryngeal cancers with low solCD44 levels were detected by *CD44* methylation status, indicating that, together, the tests may also effectively detect disease that is inaccessible on routine physical exam. Further investigation of this marker panel is under way in our laboratory.

Our results seem to contradict Rodrigo et al. (56), who found that CD44 levels were higher in laryngeal compared with oropharyngeal tumors. This may be because membrane type 1 MMP, the main protease that cleaves CD44, is elevated in tumors that metastasize to the neck (38), whereas by immunohistochemistry, CD44 levels are lower in tumors that metastasize (29). Thus, when protease activity is high in the tissues, CD44 may be released into saliva and serum and no longer visible by immunohistochemistry on the cell membrane. Because pharyngeal cancers are known to metastasize to lymph nodes more frequently than laryngeal cancers, this may explain why we see higher levels of solCD44 in oral rinses from oral cavity/oropharyngeal cancer patients. This theory is currently under investigation in our laboratory.

Conclusions

SolCD44 in oral rinses may be a useful marker for early detection of HNSCC when combined with other markers in a panel. The test seems to detect clinically occult malignancy and premalignancy as well as disease deep in the pharynx and larynx where exam requires special skills and instrumentation. Furthermore, because collection and laboratory processing are so simple, the test could potentially be used in community outreach programs, thus reaching more individuals at risk. Although unlikely to become a useful screening test by itself, it seems to have high enough specificity to be used in a panel of markers that together could achieve optimum sensitivity and specificity. Our pilot data using methylation-specific PCR suggest that *CD44* hypermethylation may explain the low solCD44 levels seen in some HNSCC patients and, together with the solCD44 test, provide a means to detect HNSCC with very high sensitivity and specificity.

Acknowledgments

Table 5. Low solCD44 tumor characteristics

Tumor no.	Stage	Site	SolCD44 (ng/mL)	Methylation	Gargle
1	$T_1N_0M_0$	L	7.42	Yes	Good
2	$T_2N_0M_0$	OC	7.84	Yes	Good
3	$T_2N_1M_0$	OP	6.25	No	Good
4	$T_1N_0M_0$	OC	2.71	Yes	Good
5	$T_3N_2M_0$	OC	6.27	Yes	Fair
6	$T_2N_2M_0$	OP	9.98	No	Fair
7	$T_3N_1M_0$	OC	10.69	Yes	Good
8	$T_2N_0M_0$	OC	10.61	Yes	Good
9	$T_2N_0M_0$	L	6.81	Yes	Good
10	$T_1N_0M_0$	OC	3.94	Yes	Fair
11	$T_3N_0M_0$	OP	3.48	Yes	Good

Abbreviations: L, larynx; OC, oral cavity; OP, oropharynx.

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