

Short Communication

Salivary Soluble CD44: A Potential Molecular Marker for Head and Neck Cancer

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Abstract

Objective: Head and neck squamous cell carcinoma (HNSCC) is a debilitating disease which is cured only 50% of the time. If diagnosed early, survival rates could reach 80%, but there is currently no practical method for early detection. CD44 comprises a family of isoforms that, in certain tumors, are alternatively spliced and overexpressed in tissues and circulation. Here we examine salivary soluble CD44 (solCD44) expression in HNSCC patients and normal controls to determine its potential as a screening tool.

Method: We did a solCD44 ELISA on saliva from 26 HNSCC patients, 10 normal volunteers, conditioned media (CM) of 4 HNSCC cell lines, and 1 CD44-negative cell line (COS-7). Western blot was done on CM from 2 HNSCC cell lines (UMSS11B and FaDu), COS-7, 3 HNSCC, and 2 normal saliva specimens to verify ELISA antibody specificity. SolCD44

levels were significantly elevated in HNSCC patients compared with normal controls (7.85 ng/mL for HNSCC patients and 1.09 ng/mL for normal controls, $P < 0.001$).

Results: The test detected 79% of mucosally invasive HNSCC using preliminary cutoff points. SolCD44 levels did not vary significantly with tumor size, stage, recurrence, history of radiation treatment, or tobacco and alcohol risk factors. A 65 to 75 kDa band, corresponding to solCD44, was detected in all of the HNSCC cell line CM and saliva whereas normal samples showed a fainter band or were undetectable.

Conclusion: In this preliminary analysis, the salivary solCD44 ELISA seems to effectively detect HNSCC at all stages. Further study is indicated because early detection is clearly important in this disease. (Cancer Epidemiol Biomarkers Prev 2005;14(3):735-9)

Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for >95% of all head and neck malignancies (1). Unfortunately, the majority of HNSCC patients present with advanced stage disease requiring multimodality therapy (2-5). Even with combinations of intensive chemotherapy, radiotherapy, and surgery, cure rates are only 30% for advanced stage disease (2, 5, 6). Those cured often face serious morbidities including speech and swallowing problems, disfigurement, and exorbitant healthcare costs. With nearly 40,000 incident cases per year, this disease poses a serious health concern in the United States (7).

In this report, HNSCC includes squamous cell cancers of the oral cavity, oropharynx, larynx, and hypopharynx. HNSCC prognosis varies by site with laryngeal cancer patients having the best and hypopharyngeal patients having the worst prognoses (8). HNSCC is classified as stage I-IV based on the American Joint Committee on Cancer tumor-node-metastasis staging system (9). Cure is achieved in >80% of stage I patients and >60% of stage II patients. For patients with more advanced disease (stages III and IV), cure is attained in <30% (6).

Because HNSCC is rarely diagnosed in stages I and II, when prognosis is good, early detection through screening is an obvious solution (10). However, screening for head and neck

cancer by clinical exam has not proven effective to date (11-14). Efforts are now focusing on molecular approaches to early detection. One candidate molecular marker for HNSCC is CD44. CD44 comprises a family of isoforms expressed in many cell types (15-17). These isoforms arise from alternative splicing of a variable exon region present in CD44 mRNA (18). Some isoforms such as CD44 standard exist in normal cells, whereas others termed CD44 variant isoforms (CD44v), are primarily expressed in tumors (19, 20). CD44 proteins are also released in soluble form (solCD44) via proteases (21) and are detectable in normal circulation (22-28). Circulating levels of solCD44 correlate with metastases in some tumors (25, 26).

Studies on solCD44 have been few and limited to plasma (27, 28). When plasma levels of a specific isoform, solCD44v6, were measured in HNSCC patients and controls, no significant difference was seen (28). Most circulating CD44v6 proteins were derived from normal epithelial compartments, including breast cells, colon cells, and squamous cells (28). To our knowledge, there are no data on total solCD44 isoforms in saliva of patients with HNSCC. We did a preliminary study on saliva from 26 HNSCC patients and 10 normal volunteers to determine if a solCD44 ELISA test can be done on saliva and is effective in detecting HNSCC.

Materials and Methods

Subject Characteristics. Twenty-six HNSCC patients and 10 normal controls were obtained according to the protocol approved by the Institutional Review Board. Control subjects were volunteers from healthcare and research fields. To decrease the potential for false-negatives in our control group, all were nonsmokers for at least 10 years. All subjects

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completed a written consent prior to enrollment. Patient characteristics are shown in Table 1. These included four patients who denied history of smoking or drinking. All patients had biopsy proven newly diagnosed or recurrent squamous cell carcinoma of the oral cavity, oropharynx, larynx, and hypopharynx.

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

Cell Culture. We obtained FaDu (hypopharyngeal carcinoma) and Cos-7 (CD44-negative cell line) from the American Type Culture Collection (Manassas, VA). MDA-1483 (oral cavity cancer) was a gift from Dr. Mien-Chie Hung, University of Texas M.D. Anderson Cancer Center (Houston, TX). UM-SCC-9 (oral tongue SCC) and UM-SCC11B (hypopharynx SCC) were gifts from Dr. T.E. Carey, University of Michigan (Ann Arbor, MI). FaDu, Cos-7, and MDA-1483 were grown in RPMI medium. UM-SCC-9 and UM-SCC-11B were grown in DMEM medium. All cell line media were supplemented with 10% fetal bovine serum, streptomycin, and penicillin. At $\sim 60\%$ confluence, cultures were washed and incubated in serum-free media supplemented with insulin, transferrin, and selenium. These conditioned media (CM) were collected at 48 to 72 hours.

Salivary SolCD44 ELISA. We measured levels of solCD44 using an ELISA assay (Bender MedSystems, Vienna, Austria) that recognizes all solCD44 normal and variant isoforms (total solCD44). This assay has been used extensively in serum and other body fluids (24, 26, 28). To our knowledge, it has not been used to study saliva of HNSCC patients. The principles of the test involve a sandwich-type ELISA where a monoclonal anti-solCD44 antibody, adsorbed onto micro-wells, 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.

Samples were vortexed, centrifuged at $3,000 \times g$, and the supernatant was used for study. The manufacturer's protocol was followed with slight modifications. The recommended dilution of 1:60 resulted in nondetectable levels of solCD44. Instead, we did the test as directed at full and half concentration for each sample. In an effort to correct for patients' varying hydration status, we normalized the solCD44 levels to protein. We did the protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol using saliva samples at full and half concentration. All samples were done in triplicate.

Statistical Analysis. We performed the solCD44 ELISA on saliva from 26 HNSCC patients, 10 normal controls, 4 HNSCC cell lines, and 1 CD44-negative cell line. Characteristics and solCD44 results for both patients and normal volunteers were entered into a computer database. Statistical analyses were done using programs of the SAS Institute, Inc. (version 8.2). The protein and solCD44 concentrations for each sample were averaged and SD calculated. The triplicate solCD44 levels for each sample were divided by the average protein concentration for that sample. The normalized solCD44 levels were then averaged and SD determined. We compared solCD44 and normalized solCD44 levels between normal volunteers and cancer patients and between specific subgroups of cancer patients based on characteristics such as stage, site, and tumor size. Student's *t* test was used in most cases as only two groups were compared. ANOVA was used to compare solCD44 levels by site (oral cavity, oropharynx, larynx, and hypopharynx) and size (T_0 - T_4).

Western Blot Analysis. To verify the specificity of the anti-solCD44 antibody used in the ELISA test, we performed Western blot on CM from two HNSCC cell lines (UMSS11B and FaDu), a CD44-negative cell line COS-7, three HNSCC,

Table 1. Patient characteristics

| Patient | Site | Tumor size* | Node* | Metastasis* | Stage* | Recurrence | Radiation | Risk Factors [†] | SolCD44 (ng/mL) \pm SD |
|---------|-----------------------|-------------|-------|-------------|--------|------------|------------------|---------------------------|--------------------------|
| 1 | larynx | 4 | 0 | 0 | IV | no | no | yes | 11.8 \pm 3.8 |
| 2 | oropharynx | 2 | 0 | 0 | II | no | no | no [‡] | 12.5 \pm 0.7 |
| 3 | oral cavity | 4 | 0 | 0 | IV | yes | yes | yes | 2.8 \pm 0.4 |
| 4 | oral cavity | 2 | 2 | 0 | IV | yes | no | no [‡] | 0.8 \pm 0.4 |
| 5 | larynx | 1 | 0 | 0 | I | no | no | yes | 3.8 \pm 1.1 |
| 6 | hypopharynx | 4 | 0 | 0 | IV | yes | yes | yes | 5.4 \pm 0.2 |
| 7 | oral cavity | 3 | 1 | 0 | III | no | no | yes | 12.4 \pm 1.5 |
| 8 | hypopharynx | 4 | 0 | 0 | IV | no | no | yes | 5.2 \pm 1.1 |
| 9 | larynx | 1 | 0 | 0 | I | no | no | yes | 2.2 \pm 0.2 |
| 10 | larynx | 4 | 1 | 1 | IV | yes | yes | yes | 6.6 \pm 0.7 |
| 11 | lymph node metastasis | 0 | 1 | 0 | III | yes | no | yes | 14.0 \pm 2.0 |
| 12 | oral cavity | 2 | 0 | 0 | II | yes | yes | yes | 3.8 \pm 1.0 |
| 13 | larynx | 1 | 0 | 0 | I | no | no | yes | 7.9 \pm 0.3 |
| 14 | hypopharynx | 3 | 2 | 0 | IV | no | no | yes | 1.6 \pm 0.4 |
| 15 | hypopharynx | 4 | 2 | 0 | IV | no | no | yes | 1.8 \pm 0.4 |
| 16 | oral cavity | 1 | 0 | 0 | I | no | yes [§] | yes | 16.5 \pm 1.5 |
| 17 | larynx | 0 | 0 | 0 | 0 | no | no | yes | 2.0 \pm 0.3 |
| 18 | oropharynx | 1 | 0 | 0 | I | no | no | yes | 4.2 \pm 0.2 |
| 19 | oral cavity | 4 | 0 | 0 | IV | no | no | yes | 5.5 \pm 0.6 |
| 20 | oral cavity | 4 | 2 | 0 | IV | no | no | yes | 14.7 \pm 2.3 |
| 21 | hypopharynx | 4 | 0 | 0 | IV | no | yes [§] | yes | 9.7 \pm 1.0 |
| | larynx | 1 | | | | | | | |
| 22 | larynx | 4 | 2 | 0 | IV | no | no | yes | 2.5 \pm 0.8 |
| 23 | larynx | 4 | 0 | 0 | IV | no | no | yes | 5.6 \pm 1.1 |
| 24 | larynx | 4 | 0 | 0 | IV | no | no | yes | 0.6 \pm 0.6 |
| 25 | oropharynx | 2 | 2 | 0 | IV | yes | yes | no | 40.0 |
| 26 | oropharynx | 1 | 1 | 1 | IV | no | no | no | 10.2 \pm 1.3 |

*Tumor, node, metastasis, and stage based on American Joint Committee on Cancer criteria (9).

[†]Risk factors refer to history of tobacco and/or alcohol use.

[‡]Social alcohol use only.

[§]This is a second primary for the patient.

and two normal saliva specimens. Proteinase inhibitors were added to fresh saliva specimens (normal and tumor). All samples were vortexed, centrifuged, normalized for protein content, and subjected to electrophoresis on a 12% SDS-polyacrylamide gel under reducing conditions. Proteins were transblotted onto nitrocellulose membranes (Protran, pure nitrocellulose transfer and immobilization membrane, Schleicher & Schuell BioScience, Inc., Keene, New Hampshire). All washes were done with TTBS (20 nmol/L Tris, 500 nmol/L sodium chloride, 0.1% Tween 20) following standard Western blotting protocol. The membrane was blocked with 5% milk. Anti-solCD44 (anti-CD44s by Bender Medsystems), was incubated with the membrane overnight at a concentration of 1:3,000 with 5% milk in TTBS. The secondary antibody (anti-mouse IgG and biotin conjugate) was applied diluted 1:1,000 with TTBS and incubated 1 hour. The membrane was treated with streptavidin-biotinylated alkaline phosphatase complex (amplified alkaline phosphatase immun-blot assay kit, Bio-Rad) followed by addition of color development solution (AP conjugate substrate kit, Bio-Rad).

Results

ELISA. Mean values for the solCD44 test were 7.85 ng/mL for HNSCC patients and 1.09 ng/mL for normal controls ($P < 0.001$). Mean values for normalized solCD44 test were 10.76 ng/mg protein for HNSCC patients and 1.80 ng/mg protein for normal controls ($P < 0.001$). SolCD44 levels did not vary significantly with stage (stages I, II, III versus stage IV; $P = 0.96$), recurrence ($P = 0.52$), history of radiation treatment ($P = 0.29$), tobacco and alcohol risk factors ($P = 0.34$), site ($P = 0.07$), or size ($P = 0.56$). SolCD44 and normalized solCD44 levels for all HNSCC patients and normal volunteers are shown in Fig. 1.

The mean protein concentration was significantly higher in the tumors than the normal controls (0.97 mg versus 0.61 mg; $P = 0.05$). This result contradicts results of other studies (29, 30). However, in a study conducted in India (30), staging was not addressed and most of the tumors were located in the buccal mucosa (an uncommon site in the United States), which may obstruct secretions from the parotid duct. Specific proteins (hyaluronidase) are elevated in saliva of tumor patients compared with controls in another study (29). If protein concentrations are higher in HNSCC patients compared with normal controls for reasons other than hydration, normalized solCD44 levels in HNSCC may be falsely lowered. For this reason, we examined both solCD44 concentrations and normalized solCD44 concentrations.

We do not have enough data to determine the appropriate cutoff point for the salivary solCD44 test. To determine the preliminary cutoff point for solCD44, we used the highest observed mean from the normal controls plus its SD (i.e., $1.74 + 0.96 = 2.7$). Similarly, to determine the cutoff point for normalized solCD44, we used the highest observed mean from the normal controls plus its SD (i.e., $3.64 + 0.69 = 4.3$). HNSCC was considered detected if the saliva specimen had solCD44 >2.7 ng or normalized solCD44 levels >4.3 ng/mg protein. With this assumption, the test detected 77% of all HNSCC samples studied and 79% of invasive HNSCC. By site, the test detected 10 of 11 oral cavity and oropharynx cancers, 9 of 13 hypopharynx and larynx cancers, and 1 of 2 HNSCC without mucosal invasion. Grouped in this manner, solCD44 levels in patients with oral cavity/oropharyngeal cancers were significantly higher than patients with laryngeal/hypopharyngeal cancers (mean 11.22 ± 3.3 versus 4.97 ± 0.9 ; $P < 0.05$). Because a screening test is most useful if it detects early disease, we looked at early disease alone. The test detected 86% of stage I and II cancers and 80% of stage I disease. For analysis of early disease, we did not include the patient with carcinoma *in situ* (SCC17) because this progresses to invasive

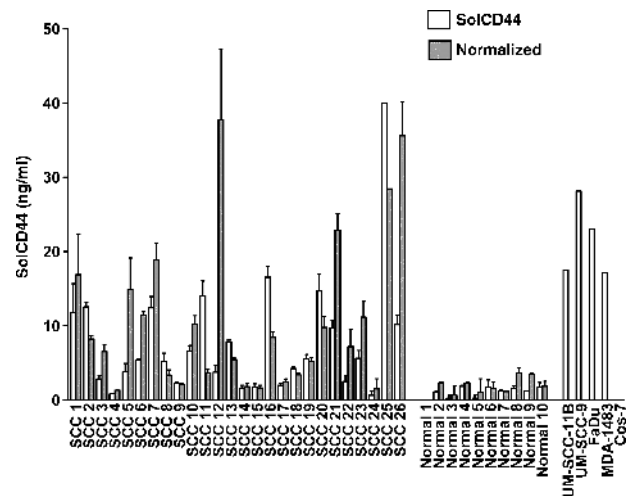


Figure 1. SolCD44 and normalized solCD44 levels for HNSCC patients and normal controls. Bars, SD. All triplicate levels for SCC25 were higher than the highest standard concentration. Exact values and therefore SD could not be calculated. All solCD44 levels below the lowest standard concentration were considered zero. The triplicate levels for Normal 1 fell below the lowest standard concentration and therefore no SD was calculated.

HNSCC $<50\%$ of the time (31). SolCD44 ELISA tests done on cell lines showed that CD44 was secreted by the HNSCC cells (Fig. 1). This suggests that solCD44 in the saliva is secreted, at least in part, by epithelial cells. The CD44-negative COS-7 cell line did not show any solCD44 as expected.

Western Blot Analysis. Results of Western blot reveal several bands in saliva of HNSCC patients and CM of HNSCC cell lines (Fig. 2). This banding pattern is explained by proteinase-mediated cleavage of CD44 as described by Nakamura et al. (32). In their study, the CD44-negative A375 cell line was transfected with the standard form of CD44. MT1-MMP-mediated cleavage resulted in three heterogeneously glycosylated fragments with bands of 65 to 70, 50 to 60, and 37 to 40 kDa. Our Western blots show bands at 65 and 68 kDa for cell line CM and HNSCC saliva, which is within the range described by Nakamura et al. The HNSCC saliva sample SCC65 shows 40 and 50 kDa bands and all the cell lines show the 50 kDa band, also consistent with the Nakamura et al. findings. HNSCC is known to express multiple CD44 isoforms. The 30 kDa band is likely a result of proteinase-mediated cleavage of additional isoforms rather than problems with antibody specificity. This is further supported by our normal samples and the Cos-7 cell lines, which show no bands or a faint band in the 65 to 70 kDa region (Normal 20).

Discussion

The WHO delineates several criteria for effective early disease detection. (a) The disease must be common and associated with serious mortality, (b) treatment as a result of screening must improve prognosis, (c) the potential benefits must outweigh the potential harms and costs of screening, and (d) screening tests must accurately detect early disease (33). Based on our understanding of HNSCC and our preliminary results, we believe that the salivary solCD44 test may be effective for early detection of HNSCC and should be evaluated further.

HNSCC is deadly (only a 50% cure rate on average; ref. 34) and is associated with devastating morbidity due to speech and swallowing problems and disfigurement. HNSCC poses a

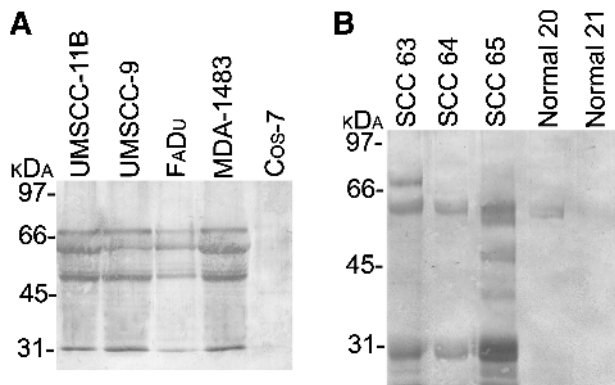


Figure 2. Western blot analysis of cell line CM (A) and saliva from HNSCC participants and normal controls (B). All the samples from HNSCC cell lines and patients show bands in the 65 to 70 kDa range as expected for the solCD44 standard (21, 32). Additional bands seen at 40 and 50 kDa have also been described (32). Because isoforms other than CD44 standard are present in HNSCC, the 30 kDa band is likely a result of proteinase-mediated cleavage of additional isoforms rather than problems with antibody specificity. This is further supported by our normal samples and the Cos-7 cell lines, which show no bands or a faint band in the 65 to 68 kDa region (Normal 20).

serious health risk with the incidence of oral cavity, pharynx, and larynx cancers in 15 of 100,000 subjects in the United States (35) and even higher incidence in other parts of the world (36). Approximately 80% of HNSCC is associated with tobacco and alcohol use (4, 6), risk for developing HNSCC is increased as high as 100 times with the heaviest use (11). Clearly, this disease meets the first of the WHO criteria for effective early detection, especially in the tobacco- and alcohol-consuming population.

Currently most HNSCC is diagnosed in stage III or IV when treatment is less effective and cure rates are 30% (2, 5, 6), largely because the only site associated with early symptoms is the larynx (12). It is estimated that if HNSCC were diagnosed in early stages (I and II), cure rates could exceed 80% (10). Furthermore, patients with earlier disease are treated with single modality therapy and have fewer problems with speech, swallowing, breathing, and disfigurement (4, 6). These significant improvements in prognosis meet the second criteria for effective early detection.

A screening test may do more harm than good (and not meet the third WHO criteria) if the detected cancer is indolent and the patient is likely to die of other causes before succumbing to the cancer (37). This conundrum does not apply to invasive HNSCC because it progresses relatively rapidly. However, HNSCC undergoes a premalignant stage similar to cervical cancer that is diagnosed histologically by various grades of dysplasia with severe dysplasia essentially equivalent to carcinoma *in situ*. Dysplasia progresses to cancer 16% to 36% of the time depending on severity (31). An indispensable screening test would detect only those dysplasias that will progress to invasive HNSCC. In this pilot study, the solCD44 test did not pick up the one patient with carcinoma *in situ*. To date, that patient has not developed invasive HNSCC after 30 months of follow-up, multiple examinations, and biopsies. Prospective studies on patients with dysplasia would help determine if salivary solCD44 predicts HNSCC progression. An accurate predictor of progression may also improve patient compliance with prevention strategies.

A final criteria for effective early detection is that the test accurately detect early disease. In this pilot study, the salivary solCD44 test detected 79% of HNSCC cases using our

preliminary cutoff points. This pilot study included seven patients with stage II or earlier invasive disease. Eighty-six percent of the early disease was identified. The solCD44 test may be more sensitive for oral cavity and oropharyngeal disease than laryngeal and hypopharyngeal disease. The test detected 91% of all oral cavity/oropharyngeal tumors and 69% of all laryngeal/hypopharyngeal tumors. The lower sensitivity observed in patients with laryngeal and hypopharyngeal cancer may be related to sampling error, as cancers located more distally in the upper aerodigestive tract are likely to have less impact on the expectorated saline/saliva mix. Further studies are under way to clarify this issue.

Our preliminary estimates of sensitivity compare favorably with other widely used screening tests such as prostate-specific antigen for prostate cancer (sensitivity 60-80%, specificity 90%; ref. 37) and the Papanicolaou test for cervical cancer (sensitivity 30-87%, specificity 86-100%; ref. 38). We are currently conducting a study to enroll more patients with early disease (stages I and II). As we accrue more subjects, we will determine a cutoff point where specificity will likely be <100%, resulting in a sensitivity that may be higher than suggested by our preliminary results.

The effects of tobacco exposure, alcohol consumption, oral health, and other medical conditions are currently under investigation in our laboratory. We suspect that saliva CD44 levels will not vary with smoking status because serum solCD44 does not vary with smoking status (27). Furthermore, 4 out of 26 HNSCC patients denied history of tobacco or alcohol exposure, yet 75% of these patients had significantly elevated solCD44 levels. The one patient who did not have elevated levels had a submucosal recurrence that was not in contact with saliva.

Other groups have contributed significantly to understanding effective early detection of HNSCC. Because oral cavity cancer is the most common site for HNSCC and it is fairly easy to examine, it seems that screening examinations would be beneficial. However, a systematic review concluded there is insufficient evidence to assess the effectiveness of community-based screening programs (39). Another tool, toluidine blue, is fairly sensitive; however, specificity for detecting early disease that progresses to cancer is limited (39). Current research focuses on molecular approaches to early detection. Hu et al. (40) recently reviewed molecular detection approaches for smoking-associated tumors. Several markers have been studied in HNSCC saliva and serum including loss of heterozygosity, microsatellite instability, and promoter hypermethylation in various panels of markers, p53 mutations, and mitochondrial DNA mutations. Lokeshwar's group showed that hyaluronic acid in saliva was elevated in 8, and hyaluronidase elevated in 11 of 11 HNSCC patients (29). As a community-based screening tool, ELISA-based techniques seem most cost-effective because they are more simple and inexpensive to perform than DNA-based techniques at this time (41, 42).

We believe that the salivary solCD44 test holds potential as a screening tool for HNSCC. To establish its worth, several objectives must be met: (a) we need to study a larger number of subjects in a target population at high risk for the disease to determine the appropriate cutoff point for the salivary solCD44 as well as the true sensitivity and specificity; (b) we should assess the ability to identify early, treatable disease; (c) we need to control for possible confounding factors including tobacco, alcohol use, and other medical conditions. We are currently conducting a study designed to meet these objectives. If the salivary solCD44 test is validated, a larger screening trial will be designed.

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