Journal of
Cancer Science & Therapy

Allegra et al., J Cancer Sci Ther 2012, 4.10
DOI: 10.4172/1948-5956.1000163

Research Article Open Access

Elisa Detection of Salivary Levels of Cd44sol as a Diagnostic Test for Laryngeal Carcinomas

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Abstract

Background: The soluble fraction of the CD44 protein (CD44sol) appears to be a possible candidate screening marker for the early diagnosis of head and neck tumors. The aim of our study was to ascertain the levels of CD44sol in the saliva of patients with laryngeal carcinoma and compare them with those of a control group of individuals to assess the reliability of the test as diagnostic marker.

Methods: Ninety-two individuals with suspected laryngeal cancer who were submitted to biopsy were selected for the study. Forty adults who underwent surgery for head and neck benign disease were recruited to form a control group. The sampling of saliva was performed on the day before the laryngeal biopsy in the patient group and on the day before surgery in the control group. CD44sol levels were detected using the ELISA method.

Results: The levels of CD44sol were significantly higher in the patient group than they were in the control group (31.4 ± 27.3 *vs.* 9 ± 7.1 ng/mL). CD44sol levels were not related to smoking and drinking habits. Analysis of the clinical data revealed an absence of significant differences between the study groups according to tumor site, histological grade, and clinical stage of T and N. The salivary levels of CD44sol were higher in advanced-stage (stages III and IV) compared with early-stage disease (43.2 ± 32.2 *vs.* 32.2 ± 20.5 ng/mL). Sensitivity and specificity were calculated based on the ROC curve and exhibited best accuracy using a predictive probability cut-off point of 10 ng/mL, with corresponding estimates of sensitivity and specificity of 89.5% and 83.3%, respectively.

Discussion: The determination of CD44sol levels in the saliva of patients with laryngeal carcinoma using ELISA seems to be a promising diagnostic test in terms of high sensitivity and specificity, low cost and noninvasiveness of the technique.

Keywords: Soluble CD44; Saliva; ELISA; Head and neck squamous cell carcinoma; Screening

Background

Head and neck squamous cell carcinomas (HNSCCs) represent about 10% and 4% of all malignancies in men and in women, respectively. In Italy, about 5,000 and 500 new cases of larynx cancer per year are reported among men and women, respectively [1].

Laryngeal cancer mainly affects people over 55 years of age and it is more common in males. The most frequent symptoms of these tumors are unexplained and persistent dysphonia (more than 2 weeks) with changes in vocal tone, pain and difficulty swallowing, persistent ear pain when swallowing, or neck adenopathy. Based on the data available, it is not possible to determine whether routine screening for laryngopharyngeal cancers, i.e., fibrolaryngoscopic examination of the oral and pharyngolaryngeal district in all smokers and drinkers older than 60 years, is as effective for individuals who do not have typical symptoms.

Recently, the idea that a simple saliva test may become an immunoassay-based screening test for the early diagnosis of tumors of the head and neck has emerged as a focus of research [2]. These studies were based on the possibility of encountering significantly higher levels of a particular antigen in the saliva of patients.

The soluble fraction of the CD44 protein (CD44sol) appears to be a possible candidate as a screening marker for the early diagnosis of head and neck tumors, because of its stem-like properties. The CD44 molecule is a type I transmembrane glycoprotein that is expressed in many cell types of mesenchymal and neuroectodermal origin. It functions mainly as an adhesion molecule and as a mediator of the cellular internalization of hyaluronic acid (HA). The interactions between HA and CD44 influence its adhesion to components of the extracellular matrix and are involved in cell aggregation, proliferation, and migration and angiogenesis. These biological properties are essential for the normal physiological activity of cells; however, in certain conditions, they are associated with pathological activity and can also be implemented in tumor cells [3]. In addition to HA, CD44 binds to fibronectins, the invariant part of the major histocompatibility complex class II (MHC-II) molecules [4], and high-molecular-weight proteoglycans [5]. The heterogeneity of these ligands is based on the fact that the gene that encodes CD44 is composed of 20 exons: the first five and last five exons are constant and the central 10 exons are subjected to alternative splicing, thus constituting the "variable region"

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Received September 02, 2012; **Accepted** September 20, 2012; **Published** September 22, 2012

Citation: Allegra E, Trapasso S, Sacco A, Aragona T, Belfiore A, et al. (2012) Elisa Detection of Salivary Levels of Cd44sol as a Diagnostic Test for Laryngeal Carcinomas. J Cancer Sci Ther 4: 330-334. doi:10.4172/1948-5956.1000163

of the receptor. The most common isoform of the receptor is CD44 standard (CD44st), which is highly expressed in hematopoietic cells. About 30 CD44 receptor variants (CD44v) have been identified, many of which appear to be expressed in tumor cells, that arise via alternative splicing at the extracellular proximal portion of the receptor. The extracytoplasmatic domain of CD44 receptors in pathological conditions/cancer is detached and it is released in biological fluids as the soluble fraction of the receptor (CD44s or CD44sol) [6].

Few studies of CD44sol in HNSCC exist in the literature; some studies considered the plasmatic levels of CD44sol, but found no significant elevation in patients compared with controls, in contrast to what was observed in tumors involving the colon and in breast cancer [7,8], presumably because of the release of different CD44sol isoforms in the plasma and the greater burden of these tumors compared with HNSCC [9]. A pilot study performed by Franzmann et al. [10] in patients with head and neck carcinoma indicated that the levels of CD44sol are seven times higher in the saliva of patients compared with controls, suggesting that the CD44sol test is highly specific for HNSCC. The presence of CD44 in the saliva seems to be a promising marker of HNSCC; in addition, this test seems to be more effective in saliva compared with the peripheral blood circulation.

Recent studies have attributed an important function to CD44 as a biomarker of a cellular subpopulation, cancer stem cells (CSCs), which exhibit characteristics of self-renewal and tumor initiation, progression, invasion, metastasis, and recurrence, as well as chemo- and radiotherapy resistance [11]. This cell subpopulation, which was isolated for the first time by Bonnet and Dick [12] using samples of acute myeloid leukemia, has also been identified in solid tumors [13-18]. In head and neck tumors, Prince et al. [19] first identified a cellular subpopulation expressing the surface marker CD44, which exhibited stem-like characteristics and was capable of reproducing after tumor implantation in immunosuppressed mice. Considering the implication of CD44 in the activation of cell replication, its antiapoptotic activity, and its potential as a marker of CSCs in epithelial tumors, we decided to study the role of CD44st in head and neck tumors by focusing on the identification of its soluble fraction in the saliva of patients with laryngeal cancer.

The aim of our study was to ascertain the levels of CD44sol in the saliva of patients with laryngeal carcinoma who were previously untreated and compare them with those of a control group of individuals, to assess the reliability of the test as a diagnostic marker.

Materials and Methods

Ninety-two individuals received at the Department of Otorhinolaryngology of the "Magna Græcia" University of Catanzaro from 2010 to 2011 for suspected laryngeal cancer and who were submitted to biopsy were selected for the study. Forty adults who underwent surgery for head and neck benign disease were recruited to form a control group. We excluded subjects who were previously treated for carcinoma of the head and neck or other malignancies and patients with a history of systemic diseases and acute or chronic inflammation of the oral cavity and salivary glands (dental abscesses, pericoronitis, gingivitis, sialadenitis, and stones in the salivary glands), patients already receiving radio- or chemotherapy, and individuals receiving pharmacological treatment with substances that can alter salivation, quantitatively and qualitatively. For each patient, clinical and anamnestic data, particularly those regarding alcohol consumption and smoking habits, were annoted in a database. To collect data on alcohol consumption, we considered one drink as containing 12 g of alcohol (one beer, 330 mL, 125 mL, one glass of wine, one shot of whiskey, 40 mL), as a reference standard. The individuals recruited were then divided into those who consumed more than two drinks per day and those who did not consume alcohol or who consumed less than two drinks per day. To collect data on smoking habits, we divided the study participants according to the number of cigarettes smoked in 24 h. Individuals who smoked >20 cigarettes per day were considered as heavy smokers, individuals who smoked ≤ 20 cigarettes per day were considered as moderate smokers, and individuals who had never smoked or had stopped smoking for more than 10 years were considered as nonsmokers.

Patients with laryngeal cancer were clinically staged using the AJCC tumor staging system [20]. The study was approved by the ethics committee of the "Magna Græcia" University of Catanzaro.

Sample collection

All individuals enrolled were asked to rinse their mouths with 2 mL of saline and to collect the saliva, without dilution, into a sterile container. The sample was immediately stored at -80°C. The sampling of saliva was performed on the day before the laryngeal biopsy in the patient group and on the day before surgery in the control group. All individuals enrolled were informed about the purpose and methods of our study and gave their informed consent before being subjected to the collection of saliva.

CD44sol ELISA

CD44sol levels were detected via sandwich ELISA (eBioscience Human scCDstd Platinum ELISA) using a commercially available ELISA kit (CD44std, soluble, human, Enzo Life Sciences, Inc., Farmingdale, NY), which recognizes all CD44 isoforms. The procedure was performed according to the instruction manual by using full-concentration samples.

The samples (20 µL) were added in duplicate to wells containing 80 µL of sample diluent. Then, HRP-conjugated antibody (50 µL) was added into individual wells, which were covered with an adhesive film and incubated (3 h at room temperature) on a rotating plate (microplate shaker) at 100 rpm. Subsequently, we emptied and washed the wells three times with wash buffer (400 µL), followed by the addition of 100 µL of tetramethylbenzidine (TMB substrate solution) to each well and incubation at room temperature for about 10 min without direct exposure to sunlight. We stopped the reaction when color appeared in the wells by adding 100 µL of stop solution. The absorbance of the test samples was obtained by spectrophotometric reading at 490 nm. We generated a CD44 duplicate standard curve for notable concentrations to seven points, with a range of values from 0.07 to 4 ng/mL. We calculated the CD44 concentration of our samples according to the relationship between absorbance and concentration of the standard curve.

Statistical analysis

The MedCalc software (version 12.2.1.0) was used for statistical analysis. The concentration of salivary CD44sol in relation to clinical anamnestic data was compared between the groups using an independent *t* test analysis with significance set at $p<0.05$. The correlation between the clinical features of patients with laryngeal cancer (location, histological grade, and T and N clinical stage) and the

salivary concentration of CD44sol was evaluated using an independent *t* test analysis if the comparison was performed between two groups, or an ANOVA test if the comparison was performed between more than two groups. Significance was set at $p<0.05$. The results were expressed as the mean ± the standard deviation (SD). The sensitivity and specificity of the tests were calculated for different cut-off points according to the analysis of a receiver operating characteristic (ROC) curve.

Results

The clinical data and salivary CD44 levels are presented in Table 1. Sixteen out of the 92 (17.4%) patients were affected by laryngeal precancerous lesions (mild dysplasia, n=7; moderate dysplasia, n=6; and severe dysplasia, n=3) and 76 out of the 92 (82.6%) patients were affected by squamous cell carcinoma of the larynx. The control group consisted of 40 patients with benign disease of the head and neck region (nasal polyps, n=10; chronic rhinosinusitis, n=10; nasal septum deviation, n=6; and polyps of the vocal cords, n=14).

The mean age of the 92 patients recruited was 64 ± 9 years; 91 patients were male and one patient was female. The mean age in the control group was 61 ± 11 years; 32 individuals were male and eight individuals were female.

The levels of CD44sol were significantly higher in the patient group than they were in the control group (31.4 ± 27.3 *vs.* 9 ± 7.1) (Table 1) and were not related to smoking and drinking in the control group. In contrast, higher CD44sol levels were found in nonsmokers and nondrinkers in the patient group.

CD44sol levels were significantly higher in patients affected by carcinoma than they were in patients with precancerous lesions and in the control group (37.2 ± 26.7 ng/mL, 6.2 ± 2.8 ng/mL, and 9 ± 7.1 ng/mL, respectively; $p<0.001$) (Figure 1). Interestingly, two individuals in the patient group with an initial histopathological diagnosis of moderate/severe dysplasia had high salivary CD44sol levels; subsequently, they were included among the patients with cancer. They exhibited salivary CD44sol levels of 25.7 ng/mL and 39.4 ng/mL, respectively. CO_2 laser cordectomy of the surgical specimen led to the establishment of a histopathological diagnosis of laryngeal carcinoma.

The localization of laryngeal cancer was glottic in 48 out of 76 cases (63.2%) and supraglottic in 28 out of 76 cases (36.8%). Clinical TNM staging was T1 in 30 out of the 76 cases (39.5%), T2 in 28 out of 76 cases (36.8%), T3 in 14 out of 76 cases (18.4%), and T4 in 4 out of 76 (5.3%) cases. Among the 76 patients, 54 (71%) had N_0 clinical stage and 22 (29%) had N_+ clinical stage.

Parameters	Controls CD44sol ng/mL No. (%) mean (SD)	Cases CD44sol ng/mL No. (%) mean (SD)	*p* value
Sex			
Male	32 (80%) 8.8 (7)	89 (96.7%) 32.2 (27.4)	0.001
Female	8 (20%) 9.9 (8.6)	3 (3.3%) 7.4 (4.4)	0.6693
***p* value**	0.79	0.1225	
Smoking habits			
Strong >20 cig/day	30 (75%) 10.1 (6.3)	64 (69.5%) 35.2 (28)	0.001
Moderate ≤ 20 cig/day	6 (15%) 1.5 (0.3)	18 (19.5%) 14.5 (11.8)	0.0773
Never or ex-smokers >10 (years)	4 (10%) 12.1 (14)	10 (11%) 37.1 (33)	0.3314
***p* value**	0.125	0.013	
Drinking habits			
>2 drinks/day	18 (45%) 9.3 (7.3)	46 (50%) 25.6 (14.8)	0.0023
No or ≤ 2 drinks/day	22 (55%) 8.8 (7.2)	46 (50%) 37.2 (35)	0.0102
***p* value**	0.8797	0.0413	
Total	40 9 (7.1)	92 31.4 (27.3)	0.0004

Table 1: Demographic data, risk factors, and CD44sol data for cases and controls.

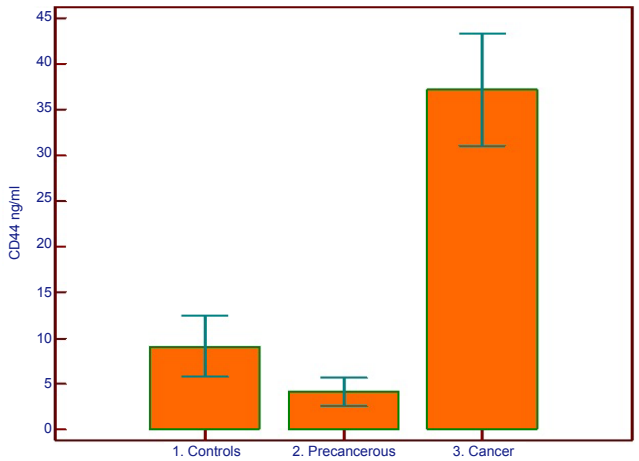

Figure 1: Mean CD44sol levels of the groups of individuals enrolled: control, dysplasia, and cancer groups.

Parameters	No. (%)	CD44sol ng/mL mean (SD)	*p* value
Site			
Supraglottic	28 (36.8%)	35.6 (30)	0.703
Glottic	48 (63.2%)	38 (24.9)	
Histological grade			
Well differentiated	10 (13.1%)	42.5 (29)	0.763
Moderately differentiated	46 (60.5%)	37 (22.7)	
Poorly differentiated	20 (26.4%)	34.8 (34)	
Tumor size			
T1	30 (39.5%)	32.3 (23.3)	
T2	28 (36.8%)	39.7 (29)	0.461
T3	14 (18.4%)	44.6 (30)	
T4	4 (5.3%)	29.9 (22.4)	
Lymph node status			
pN0	54 (71%)	35.6 (24.5)	0.446
pN_+	22 (29%)	40.8 (31.9)	
Clinical stage			
Early clinical stage (I–II)	42 (55.3%)	32.3 (20.5)	0.09
Advanced clinical stage (III–IV)	34 (44.7%)	43.2 (32.2)	

Table 2: Mean levels of salivary CD44sol according to tumor features.

The analysis of the clinical data in relation to the concentrations of salivary CD44sol revealed an absence of significant differences between the study groups according to tumor site, histological grade, and clinical stage of T and N (Table 2).

CD44sol salivary levels were higher in advanced-stage disease (stages III and IV) than they were in early-stage disease (43.2 ± 32.2 *vs.* 32.2 ± 20.5 ng/mL). However, this difference did not appear to be significant ($p=0.09$) (Figure 2).

Sensitivity and specificity values derived from various predicted probability cut-off points based on the ROC curve are summarized in Figure 3. Best accuracy was obtained using a predictive probability cut-off point of 10 ng/mL, with corresponding estimates of sensitivity and specificity of 89.5% and 83.3%, respectively, with six false-positive and eight false-negative values. At cut-off points greater than 17.4 ng/mL, the sensitivity and specificity of the test were 76.3% and 91.6%,

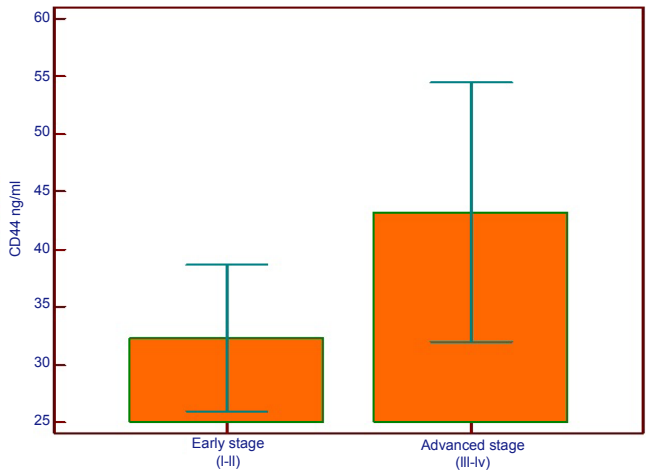

Figure 2: CD44sol levels in the group of tumors according to clinical stage (early and advanced).

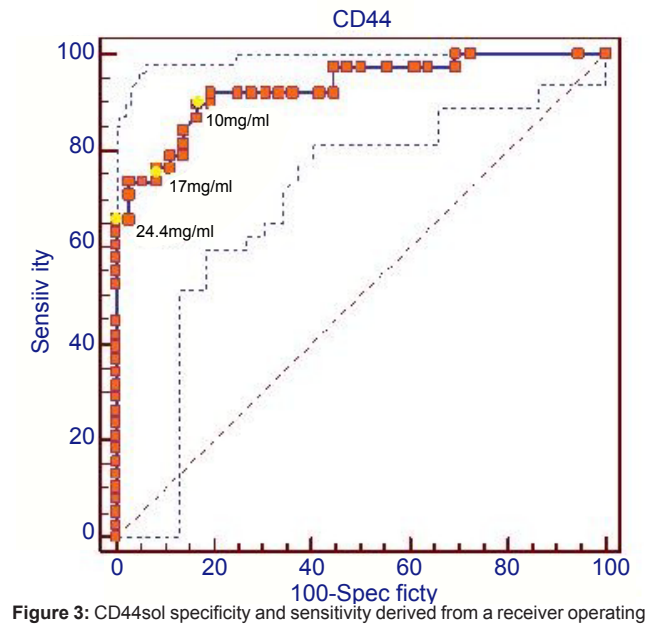

Figure 3: CD44sol specificity and sensitivity derived from a receiver operating characteristic (ROC) curve at different cut-off points.

respectively, with three false-positive and 18 false-negative values. At cut-off points greater than 24.4 ng/mL, the sensitivity and specificity of the test were 65.7% and 100% respectively.

Discussion

Studies on the use of enzyme immunoassays for the detection of CD44sol in saliva for diagnostic purposes in head and neck cancers are scarce [10-22]. Unlike other reports, our study was limited to laryngeal sites exclusively, which was intended to determine the specificity of the test according to the laryngeal site and to limit the possible discrepancies caused by the well-known biological diversity of the head and neck tumors, as reflected by the variation of clinical behavior according to location.

Data are conflicting regarding the expression of CD44 in head and neck tumors in relation to their subsite. In carcinomas of the oral cavity and oropharynx, the evidence available suggests that low expression of CD44 correlates with increased metastasizing capacity and poor prognosis [23-26]. Conversely, in laryngeal carcinomas, the high expression of CD44 appears to correlate with poor prognosis because of metastasis (regional or distant) and radiotherapy resistance [27-29].

In our study, the levels of CD44sol detected in the saliva of patients with laryngeal cancer were significantly higher than they were in patients with precancerous lesions and in the control group.

We must emphasize the relevance of the results obtained for two patients with an initial histopathological diagnosis of dysplasia, who had high levels of CD44sol and whose final diagnosis of the surgical specimen was cancer. These data seem to correlate with the role of the CD44 antigen as a marker of CSCs in laryngeal tumors.

According to the hierarchical theory [30] of tumor development, CSCs represent a cell population that fuels tumor growth, confers radio- and chemotherapy resistance, and promotes local and distant metastasis. The remaining cellular components of the tumor mass would consist of aberrant differentiated cells that have lost the ability to replicate [17].

The finding of high CD44sol levels in the saliva of patients affected by cancer might be related to the amount of the extracellular fraction of the CD44 antigen released by stem cells that remain on the tumor mass. Despite we did not find correlations between CD44sol levels and the independent factors grade and T and N stage; however, we found that high CD44sol levels correlated with advanced disease, probably because of the increased presence of CSCs in advanced stages of disease. We are currently conducting a study to test this hypothesis.

In their study, Franzmann et al. [10,21,22] considered various subsites of the head and neck region and reported lower average levels of CD44 compared with those encountered here in the group of patients with laryngeal carcinoma. However, this difference might be due to methodological differences in the collection of the sample (i.e., oral rinse *vs.* whole saliva).

Based on our results, the diagnostic power of CD44sol determination in the saliva of patients with laryngeal carcinoma seems promising and not influenced by risk factors. The CD44sol levels determined in the control group were not significantly different regarding smoking and drinking, which is in agreement with the results of Franzmann et al. [10,21,22]. This finding was confirmed in the patient group, in which we found surprisingly high levels of CD44sol in nonsmokers and nondrinkers, despite the observation that the majority of the patients were smokers and half of them were drinkers, reflecting the incidence of smoking and alcohol use in the Italian population.

The observed sensitivity of ELISA ranged from 65.7% to 89.5% and its specificity ranged from 83.3% to 100%, depending on the cut-off value selected. These data are superior to those obtained in other studies using various markers and different methods of investigation, such as loss of heterozygosity [31], methylation-specific markers [32], telomerase activity [33], mitochondrial DNA mutations [34] and, recently, multiplexed immunobead-based technology [35]. Those investigations reached a sensitivity that ranged from 35 to 84.5% and a specificity that ranged from 30 to 98% and required long run times, skilled personnel, and expensive technology. Moreover, the technology and skilled personnel are not widely available; thus, they represent inadequate conditions for an ideal screening method.

The determination of CD44sol levels in the saliva of patients with

laryngeal carcinoma using ELISA, compared with other screening tests (such as PSA for prostate cancer [36] and the Pap test for cervical cancer [37] seems to be a promising diagnostic test in terms of high sensitivity and specificity, low cost and noninvasiveness of the technique.

Conclusion

The determination of CD44sol levels as a potential diagnostic marker of laryngeal cancer in the saliva of patients with laryngeal carcinoma using ELISA seems to be a promising method. However, additional and larger studies are needed to confirm these results.

Competing Interests

The authors declare that they have no competing interests. Non-financial competing interest.

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