Acta Scientific Otolaryngology (ISSN: 2582-5550)

Volume 6 Issue 1 January 2024

Research Article

Expression of CD44 and its Clinicopathological Correlation in Oral Squamous Cell Carcinoma: An Immunohistochemical Study

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DOI: 10.31080/ASOL.2024.06.0627

Received: July 31, 2023

Published: December 28, 2023

Abstract

Aim: CD44 expression has been associated with aggressive behavior in cancers of different types. Although CD44 is a potential prognostic marker, it has not been adopted to wider clinical use in OSCC patients. The study aims at the assessment of the immunohistochemical expression of CD44 in Oral Squamous Cell Carcinoma (OSCC) and to correlate its expression with prognostic parameters.

Methodology: A total of 36 cases of OSCC, were included in the study which included well differentiated squamous cell carcinoma (WDSCC) and moderately differentiated. The sections were subjected to immunohistochemical study using CD44 antibody. The degree of intensity and distribution of CD44s immunostaining was assessed and correlated with prognostic markers such as tumor stage (tumor size), tumor grade (Broder's histological grading), tumor site, nodal status, and survival.

Results: CD44 expression by tumor cells in OSCCs is statistically correlated with overall survival and tumor grade i.e. stronger intensity of CD44 immunoexpression was observed in WDSCC group, followed by MDSCC group. There was a correlation of CD44 with overall survival but no statistical significance observed with respect to the other prognostic markers.

Conclusion: Based on these observations it can be suggested that the decrease in expression of CD44 in OSCC cells may be due to the reduced cell-to-cell and cell-to-matrix adhesion, resulting in easy detachment from the rigid constitution. Low expression of CD44 in OSCC tissues may be an indicator of tumor invasion and high metastatic potential.

Keywords: CD44; Oral Cancer; Squamous Cell Carcinoma; Immunohistochemistry

Introduction

Squamous cell carcinomas encompass at least 90% of all oral malignancies. Oral cancer holds the eighth position in the cancer incidence ranking worldwide, with epidemiologic variations between different geographic regions (it is the third most common malignancy in south-central Asia). Cancer cells result from disruptions in circuits that regulate proliferation and homeostasis of normal cells. Every cell type has a unique molecular signature, referred to as biomarkers, which are identifiable characteristics such as levels or activities (the abilities of genes or proteins to perform their functions) of a myriad of genes, proteins or other molecular features. Biomarkers are therefore, an objective measure or evaluation of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention. The CD44 proteins form widely expressed family of adhesion molecules involved in cell-cell and cell-matrix interactions. It was shown that aberrant expression of CD44 and its variant forms is associated with invasive and metastatic potential of cancer cells as well with poor prognosis in several types of human cancer. The CD44 and its isoforms have been detected in many types of normal human tissue, predominantly in the skin, gingiva and tongue, larynx, esophagus, cervix, bladder, pancreas, and parotid gland. Multiple CD44 isoforms are expressed by normal stratified squamous epithelia, such as the epidermis and the lining of the oral cavity. Numerous studies on histopathologic features of tumor and host response parameters in OSCC have shown variable prognostic significance. Not just analysing the immunoreactivity of biomarkers, the surgeons role is mainly to correlate these immunohistochemical findings with the clinicopathological parameters such as Age/Gender, Tumor site, T stage, N stage, M stage, Histological grade, Clinical stage, Habit, Tumor thickness, CD44 positivity, Survival/prognosis, Recurrence, Other biomarkers used and Variants/Isoforms.

Research question

Is there any correlation between the expression of CD44 and clinicopathological parameters and prognosis in patients with OSCC?

Thus, the Aim of our study was to investigate the role of CD44 immunoexpression in determining the prognosis of oral squamous cell carcinoma with the objectives of assessing CD44 expression and correlating it with clinicopathological parameters (tumor size, lymph node metastasis, degree of differentiation) and prognosis.

Materials and Methods

The present study comprises of 36 cases (retrospective and prospective)of OSCC who have undergone surgical resection and neck dissection with or without reconstruction by the Department of Oral and Maxillofacial Surgery, FDS, RUAS, and Department of Surgical Oncology, Ramaiah Teaching and Memorial Hospitals, Bengaluru, from July 2012 to May 2017. Formalin-fixed paraffin-embedded tissue blocks and histopathology reports were retrieved from the archives of Department of Pathology, Ramaiah Teaching and Memorial Hospitals. These cases were evaluated immunohistochemically for the expression of CD44.

Inclusion criteria

FFPE blocks and case records of patients with histologically proven primary OSCC who underwent surgical resection of primary tumor with neck dissection with or without reconstruction.

Exclusion criteria

Incomplete case records, Cases whose primary tumor blocks were unavailable, Cases of recurrent OSCC.

Ethical approval

Ethical approval was taken from the institutional review board and ethics committee.

These cases were histologically confirmed and graded by using Broder's grading system. These cases were evaluated immunohistochemically for the expression of CD44 using monoclonal antibody (BioCare Medicals, USA) with BioCare polymer and HRP detection system.

IHC staining procedure: 4um thick sections of all FFPE blocks were mounted onto polylysin coated slides and these were incubated at 58 degree C overnight. The sections were deparaffinized in 2 changes of xylene for 15 minutes each, rehydrated through a series of graded alcohol and distilled water. Antigen retrieval were performed by microwave method under EDTA buffer (pH-8.0) for 30 minutes at 100 degrees. The slides which were treated with antigen retrieval solution were allowed to cool to room temperature. The slides were rinsed in distilled water for 5 minutes and transferred into Tris buffer solution (pH-7.5) for 10 minutes. Blocking of endogenous peroxide were done be flooding the slides with 3% hydrogen peroxide for 5 minutes, followed by washing in 2 changes of

TBS buffer for 5 minutes each followed by snipper for 10 minutes to block nonspecific reaction with other tissue antigen. Snippers were drained and sections were flooded with CD44 primary antibody in dilution of 1:100 for about 60 minutes respectively. This were followed by 2 changes of TBS buffer wash for 5 minutes to remove unbound antibodies. Super enhancer was used for 30 minutes to enhance reaction between primary and secondary antibodies.

Immunostaining was evaluated and scored by 2 pathologists for CD44 expression. The most representative tumor areas were selected for scoring the immunostaining pattern. The degree of positive staining for CD44s antibody was evaluated in cell membranes of tumor cells.

The staining intensity of the CD44 was considered as

- Negative (-)
- Weak (+)
- Moderate (++)
- Strong (+++) (Hema., *et al*. 2014)

Percentage of cells (distribution)

- 0-25% - GRADE 1
- 26-50% - GRADE 2
- 51-75% - GRADE 3
- >75% - GRADE 4.

The degree of positive staining for CD44s antibody was calculated by universal wellestablished semiquantitative intensity grading system. (Hema., *et al*. 2014) As this gave only the qualitative data which was very subjective, a modified grading system with the percentage distribution of cells was used to obtain quantitative values. 1 to 4 for intensity (I) such as negative, mild, moderate and strong; and Distribution (D) as % distribution of cells. Tissues with I × D less than or equal to four were considered weakly positive and those with I × D greater than four were designated strongly positive [7].

Statistical Analysis - Statistical analysis was done using SPSS version 22.0 software. The association of various parameters was done using Chi square test. P value ≤0.05 was considered as statistically significant.

Results

A total of 36 patients with histologically proven OSCC consisting of 13 men and 23 women were included in this study. Patient age ranged 35–80 years (mean, 56.13 ± 21.1 years).

The site of OSCC in the patients included the and buccal mucosa (19 cases; 52.8%), gingivobuccal sulcus (11 cases; 30.6%), and tongue (6 cases; 16.7%). The followup period in all patients was in the range of 6 months to 4 years. In all cases, resection of primary tumor, neck dissection with or without reconstruction was performed.

Tumor size is an important determinant of surgical planning (whether the tumor is resectable or not; and involvement of vital structures). In the present study this association between CD44 expression and clinical and pathological tumor size was not found to be statistically significant ($p > 0.05$).

Nodal status is important to assess the extent of the disease, its metastatic potential and its presence dictates the use of adjuvant chemo radiotherapy following surgical resection. In the present study this association between CD44 expression and clinical nodal status was not found to be statistically significant ($p > 0.05$).

14 out of 26 cases which expressed strong immunopositivity of CD44 were well differentiated squamous cell carcinomas and 12 out of 26 cases expressing strong positivity were moderately differentiated squamous cell carcinomas. Similarly, weakly positive staining was seen in 5 out of 10 cases of WDSCC and 5 out of 10 cases of MDSCC. This association of CD44 expression and degree of differentiation was not statistically significant ($p = 0.83$). Stage of the disease – 11 out of 26 patients who showed strong expression of CD44 had stage IV disease whereas 6 out of 10 patients who showed weak expression of CD44 had stage IV disease and this association of CD44 expression with stage of the disease was not statistically significant.

The analysis of overall patient survival rates in terms of clinicopathological tumor features revealed some significant results. Enhanced CD44 expression was associated with better survival rates, but this finding was not statistically significant ($p = 0.82$). 20 out of 26 cases which showed strong CD44 expression were alive which implies that stronger the expression better is the overall survival and better is the prognosis. Among cases which showed weak positivity, 2 were deceased which implies that weaker the expression of CD44, poorer is the overall survival rate.

Case no	Tumor site	Tumor size (clinical)	Nodal status (clinical)	Overall stage	Degree of differentiation	CD44 immunopositivity
1	Gbs	T2	N0	Stage I	WDSCC	Strongly positive
2	Buccal mucosa	T4	N1	Stage IVa	WDSCC	Strongly positive
3	Buccal mucosa	T2	N0	Stage I	WDSCC	Strongly positive
4	Gbs	T1	N0	Stage IVa	WDSCC	Strongly positive
5	Gbs	T2	N0	Stage III	WDSCC	Strongly positive
6	Gbs	T3	N1	Stage IVa	WDSCC	Strongly positive
7	Gbs	T2	N0	Stage IVa	WDSCC	Strongly positive
8	Gbs	T1	N1	Stage IVa	WDSCC	Weakly positive
9	Gbs	T1	N0	Stage III	WDSCC	Strongly positive
10	Buccal mucosa	T2	N0	Stage I	WDSCC	Strongly positive
11	Buccal mucosa	T4	N2	Stage IVa	WDSCC	Strongly positive
12	Tongue	T2	N0	Stage II	WDSCC	Strongly positive
13	Buccal mucosa	T3	N0	Stage III	WDSCC	Strongly positive
14	Buccal mucosa	T2	N0	Stage II	WDSCC	Weakly positive
15	Buccal mucosa	T2	N1	Stage II	WDSCC	Strongly positive
16	Buccal mucosa	T2	N0	Stage II	WDSCC	Strongly positive
17	Tongue	T2	N1	Stage III	MDSCC	Strongly positive
18	Gbs	T2	N0	Stage II	MDSCC	Strongly positive
19	Gbs	T2	N0	Stage II	MDSCC	Strongly positive
20	Tongue	T1	N1	Stage IVa	MDSCC	Strongly positive
21	Gbs	T4	N1	Stage IVa	MDSCC	Weakly positive
22	Buccal mucosa	T3	N0	Stage III	MDSCC	Weakly positive
23	Buccal mucosa	T1	N0	Stage I	MDSCC	Strongly positive
24	Tongue	T2	N1	Stage IVa	MDSCC	Strongly positive
25	Buccal mucosa	T4	N1	Stage IVa	MDSCC	Strongly positive
26	Buccal mucosa	T4	N1	Stage IVa	MDSCC	Strongly positive
27	Buccal mucosa	T2	N0	Stage II	MDSCC	Weakly positive
28	Tongue	T3	N1	Stage IVa	MDSCC	Weakly positive
29	Tongue	T2	N1	Stage IVa	MDSCC	Strongly positive
30	Gbs	T2	N1	Stage IVa	MDSCC	Weakly positive
31	Buccal mucosa	T2	N0	Stage IVa	MDSCC	Weakly positive
32	Buccal mucosa	T2	N0	Stage IVa	MDSCC	Weakly positive
33	Buccal mucosa	T4	N1	Stage IVa	MDSCC	Strongly positive
34	Buccal mucosa	T2	N0	Stage II	MDSCC	Strongly positive
35	Buccal mucosa	T1	N1	Stage III	MDSCC	Weakly positive
36	Buccal mucosa	T2	N0	Stage II	MDSCC	Strongly positive

Table 1

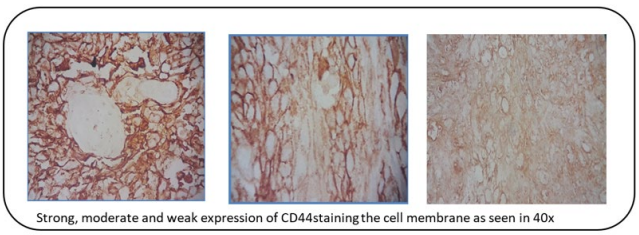
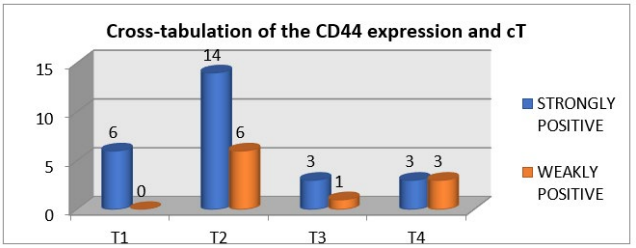


Figure 1



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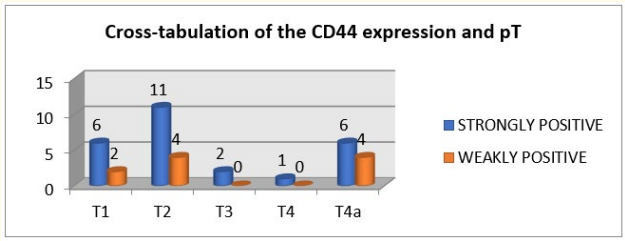

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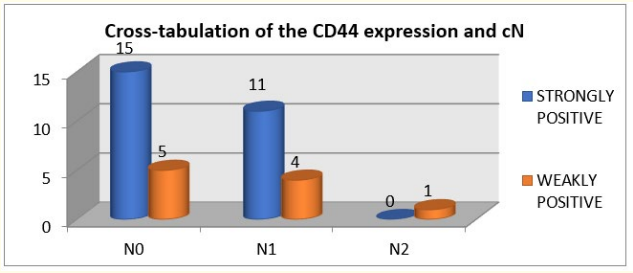

Figure 4

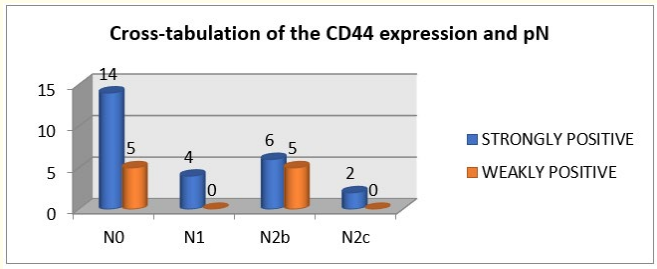

Figure 5

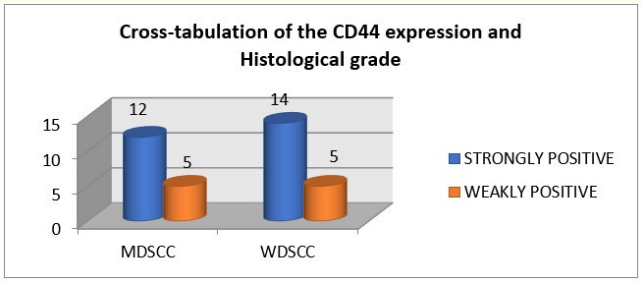

Figure 6

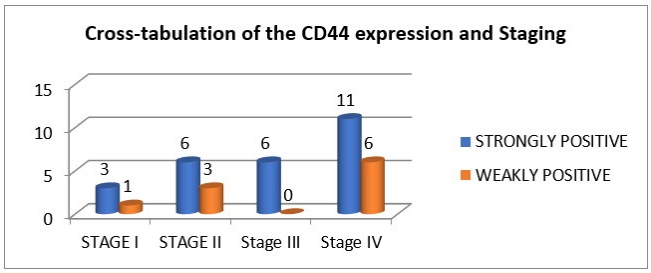

Figure 7

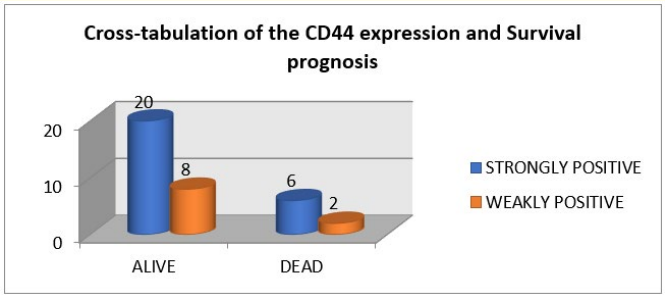

Figure 8

Discussion

Since the Cancer Stem Cell hypothesis has emerged as a key theory to explain cancer progression, the expression patterns of several pluripotent stem cell markers and transcription factors have been studied in various cancer tissues or cell lines to determine their correlation with long-term prognosis [16]. One such marker CD44 is a cell surface glycoprotein that acts as a receptor for hyaluronic acid and as an adhesion molecule. This cell surface protein plays a role in tumor cell invasion, metastasis, and angiogenesis by interacting with certain matrix metalloproteinases [16].

Numerous studies on histopathologic features of tumor and host response parameters in OSCC have shown variable prognostic significance. The main prognostic determinate in carcinomas of the oral cavity is stage of the disease. The TNM classification of cancers arising in oral cavity, based upon extent and size of primary tumor, absence/presence and extent of regional lymph node metastasis, is a generally useful and widely applied method for estimating prognosis and planning therapy [7].

In the present study, CD44, was immunohistochemically investigated in 36 patients with OSCC to identify any relationship of their expression patterns with clinicopathological tumor features and patient prognosis. The results of this study demonstrated that the immunohistochemical expression patterns of CD44 were directly associated with clinicopathological features, including tumor size, lymph node metastasis, and histological grade as well as overall survival rates.

As far as the demographics is concerned, number of females were more compared to males with a peak age range of 46-55 years. In males, the most common age group was 35-45 years and 56-65 years. There was no significant association between the age and the clinicopathological paramters and more importantly with the expression of CD44. Other studies which have taken age into consideration like [13,14,17,25] showed similar results.

Tumor site is another determinant of aggressiveness in cancers of head and neck including oral cavity. In the present study, 52.8% of the cases were involving buccal mucosa, 30.6% gingivobuccal sulcus tumors and 16.7% cases had tongue involvement. This had no significant correlation with the expression of CD44 ($p > 0.05$). This is in accordance with study conducted by Hema., *et al*. [7]. Frequency of certain locations for cancer development depends on the geographic location and types of habits associated etc. [13,17,21] showed more cases of tongue cancer compared to other sites in their study with no statistical significance. Tumor size (TNM staging).

Tumor size is an important determinant of surgical planning (whether the tumor is resectable or not; and involvement of vital structures) followed by prognosis. In the present study, 14 cases of T2 lesion followed by 6 cases of T1 lesion showed strong expression of CD44. However, 6 cases having T2 lesion followed by 3 cases of T4 lesion showed weak expression of CD44. Considering the pathological tumor size obtained from the resected specimen with negative surgical margins, 14 cases of T2 lesion followed by 6 cases of T1 lesion and 6 cases of T4a lesion showed strong expression of CD44. However, 4 cases having T2 lesion followed by 4 cases of T4a lesion showed weak expression of CD44. This association between CD44 expression and tumor size was not found to be statistically significant ($p > 0.05$). Generally, greater the tumour size, poorer is the prognosis [7].

A significant correlation was found between T classification and Lin-CD44þ cell frequency in a study (Joshua., *et al*. 2011). One important observation made in our study was that, even when tumor size was large (T4a), strong immunopositivity of CD44 was observed and these patients had better prognosis and overall survival. Thus strong expression CD44 gives better prognosis and overall survival rates. Similarly, weaker expression of CD44 even in small tumors (T2), leads to poor prognosis and such patients were deceased.

Regional lymph node status (TNM staging)

Nodal status is important to assess the extent of the disease, its metastatic potential and its presence dictates the use of adjuvant chemo radiotherapy following surgical resection. In the present study, 15 cases of N0 disease followed by 11 cases of N1 disease showed strong expression of CD44. However, 5 cases of N0 disease and 4 cases with N1 disease and 1 case of N2 disease showed weak expression of CD44. Considering the pathological nodal status obtained from the neck dissection specimen 14 cases of N0 disease followed by 6 cases of N2b disease showed strong expression of CD44. However, 5 cases of N0 disease and 5 cases with N2b disease showed weak expression of CD44. This association between CD44

expression and pathological nodal status was not found to be statistically significant (p>0.05). it is noteworthy that a patient with N2b disease showing strong expression of CD44 had better prognosis and overall survival. Thus, in the present study, strong expression of CD44 was related to higher frequency of negative neck nodes. This is in conjunction with the study conducted by Hema., *et al*. [7]. And a contradicting observation was put forth by Lee., *et al*. where they said weak expression of CD44 was significantly related to a higher frequency of negative neck node metastasis [16]. Also, in a study conducted by Piffk., *et al*. all 25cases of lymph node metastases, uniformly strong immunostaining reactions were found with both anti-CD44- v6 and -v5 antibodies. However, There was positive correlation between reduced expression of CD44v9 and metastasis to lymph nodes in tongue SCC [22].

Distant metastasis (TNM staging)

No distant metastasis was seen any of the cases.

Histological grade (Broders classification)

The decrease in the intensity of the CD44 levels with the increase in the grade of the tumor suggests reduced cell-to-cell adhesion, resulting in easy detachment of the cells from a rigid constitution. Low expression of CD44 in OSCC tissues may be an indicator of high metastatic potential and may be related to lymph node metastasis and poor prognosis [7]. In the present study, 14 out of 26 cases which expressed strong immunopositivity of CD44 were well differentiated squamous cell carcinomas and 12 out of 26 cases expressing strong positivity were moderately differentiated squamous cell carcinomas. Similarly, weakly positive staining was seen in 5 out of 10 cases of WDSCC and 5 out of 10 cases of MDSCC. This association of CD44 expression and degree of differentiation was not statistically significant (p > 0.05). Thus, lower grade (WDSCC) tumors showed strong expression of CD44 indicating better prognosis. In similar studies, Immunoreactivity of CD44v9 in the primary SCCs of the tongue did not correlate with the histological grading of the tumors [22] There was a correlation between immunohistochemical staining for CD44v9 and grading of the tumour tissue showing a statistically signifcant higher frequency of decreased CD44v9-expression in lower differentiated tumours [24]. Highgrade tumors exhibited enhanced expression of CD44, but this finding was not statistically significant [16].

Overall survival and prognosis

Patients with OSCC whose tumors had weaker expression of CD44 had less favourable clinicopathological features and lower survival rates, but no statistical significance was observed. In the present study, Enhanced CD44 expression was associated with better survival rates, but this finding was not statistically significant (p = 0.82). 20 out of 26 cases which showed strong CD44 expression were alive which implies that stronger the expression better is the overall survival and better is the prognosis. Among cases which showed weak positivity, 2 were deceased which implies that weaker the expression of CD44, poorer is the overall survival rate. The study of CD44 as prognostic factor in oral cancer has revealed divergent results showing a poor survival with the under expression of the receptor, others with overexpression of the receptor or simply with no associations to survival [4,13,15]. Interestingly, Kokko., *et al*. [13] reported significant association of CD44 in oropharyngeal, hypopharyngeal and laryngeal and laryngeal carcinomas but not in oral cancer. This was reinforced in a meta-analysis by Chen., *et al*. [4] where under expression of CD44 was related with overall survival in patients with laryngeal and pharyngeal cancer but not for oral cancer. Overexpression of CD44 in tumors has been shown to predict an increased capacity of resistance to radiation therapy and an increased risk of local recurrence. At the same time, in some studies, CD44 underexpression on tumor cells has been associated with poor prognoses, especially in squamous -cell carcinomas existing in the oral area. In a study conducted by [14] Loss of CD44-v7-8 immunoreactivity was significantly correlated with poor prognosis. The average survival time became shorter with the reduction of CD44v9 expression in cases of tongue SCC [22].

The main goal of any cancer treatment is early detection, targeted therapy to achieve better prognosis and survival. Many genetic and molecular studies are thus done along with CD44 to develop targeted therapy to regulate these genes which help in tumor progression [10].

Conclusions and Future Directions

Based on the above findings, we conclude that CD44 immunoexpression has a positive correlation with the overall survival. However there was no statistically significant correlation between the expression of CD44 and clinicopathological parameters like tumor size, regional lymph node metastasis and degree of differentiation

of the tumor. Hence, CD44 might be of prognostic value in assessing the invasive capacity and metastatic potential of oral squamous cell carcinomas. But, further studies on larger samples, using a combination of markers and by using different isoforms like v6, v4 and v9 are needed to establish the prognostic value of CD44 and its variant isoforms.

Suggestions for Future Directions

- CD44 staining with different isoforms especially CD44v9, CD44v6, CD44v4.
- To include polymerase chain reaction studies for further validation
- To include a combination of markers (one epithelial and one connective tissue marker) to predict exact behavior of OSCC (podoplanin, NANOG).

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