Original Article

Expression of stem cell biomarker CD44 in oral squamous cell carcinoma and its association with lymph node metastasis and TNM staging

ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is one of the most prevalent cancers in the world. OSCC is a highly invasive lesion frequently having soaring morbidity as well as substantial mortality, attributed to resistance to therapy, metastasis, and recurrence driven by specific populations of cancer stem cells (CSC). The evidence of the association of expression of stem cell biomarker CD44 and metastatic potential of the tumor is inconclusive in OSCC and hence needs further evaluation.

Objectives: To determine the immunohistochemical expression of CD44 in OSCC and to find its association with lymph node metastasis and TNM staging.

Materials and Methods: One hundred and five histologically proven cases of OSCC were studied. Histopathological parameters like depth of invasion, presence of lymph node metastasis, grading, and TNM staging were done according to the new AJCC staging criteria. Both intensity and proportion of CD44 expression were recorded.

Results: The mean age observed in this study was 52.59 years with a male: female ratio of 1:3.76. Forty-nine cases (46.6%) showed a depth of invasion of more than 10 mm. Fifty-two out of one hundred and five cases (49%) had nodal involvement. TNM staging was 5.7%, 7.6%, 44.7%, and 42% for stages I, II, III, and IV, respectively. The majority of the cases (87.5%) showed CD44 expression in the tumor. There was a significant association between the CD44 expression and lymph node metastases ($P < 0.001$). Higher CD44 expression was seen in stages III and IV ($P < 0.001$).

Conclusion: CD44, a stem cell biomarker is significantly associated with higher TNM stage and lymph node metastases. This may be useful in predicting the tumor behavior in the small biopsy.

KEY WORDS: Cancer stem cells, CD44, lymph node metastasis, oral cancer, squamous cell carcinoma

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a major health problem, particularly in the Indian subcontinent. Over 30% of all cancer diagnoses in the nation are head and neck malignancies, with oral cancers making up roughly half of them.[1,2] OSCC is a highly invasive lesion and is associated with high morbidity and significant mortality, and over the last few decades there has been very little improvement, both in disease-free survival and overall survival of OSCC patients.[3] Most of the morbidity and mortality can be attributed to resistance to therapy, both regional and distant metastasis and recurrence are driven by specific populations of cancer stem cells (CSC) possessing intrinsic biological properties of both stem cells and cancer cells, and thus they can reconstitute a tumor that is identical to the parent tumor.[3]

CSCs mediate both progression of the tumor as well as the development of therapeutic resistance. CSCs can be pre-existing or may develop after therapy. CSCs express drug exporters, detox proteins and can enter quiescency and become resistant to DNA damage preventing cell death.[4]

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The role of CSCs in malignancies was first demonstrated in 1994 in acute myeloid leukemia. It was in 2003 when CSCs were recognized in solid tumors inclusive of breast and brain.[5]

Over the past decade, multiple studies have been conducted and have led to the identification of certain important CSC-related biomarkers in OSCC, namely, CD44, ALDH1, CD133, OCT3/4, NANONG, and SOX2.[5] Expression of the CD44 marker has been significantly associated with local recurrence, poor differentiation, and metastatic potential of the tumor in many malignancies.[6]

So, this study aimed to evaluate CD44 as a prospective CSC biomarker to predict the neoplastic potential of the tumor.

MATERIALS AND METHODS

Patients and tissue sample

On obtaining the clearance from Institute Ethics Committee (IEC: 567/2020-21) the research was initiated. Written informed consent was taken from all participating patients in this prospective study which included 105 patients of OSCC who underwent tumor resection along with neck dissection. The sample was collected in formalin, and sections from the tumor were processed as formalin fixed paraffin embedded tissue blocks. The H and E slides from the tumor were evaluated for histopathological parameters like depth of invasion, grading, and TNM staging.

All histopathologically diagnosed cases of OSCC clinically staged between T2 and T4 were included while patients who presented as recurrence or second primaries or post neoadjuvant chemotherapy were excluded from the study.

The depth of invasion was calculated microscopically from sections having both tumors along with normal mucosa as the maximum perpendicular depth of the tumor with respect to the normal mucosa (plumb line). It was divided into three categories as followed in the 8th American Joint Committee on Cancer (AJCC) staging system: 0–5 mm, 6–10 mm, and >10 mm.[7]

The tumor was graded into well-differentiated (WDSCC) [Figure 1], moderately differentiated (MDSCC), and poorly differentiated (PDSCC), and the TNM staging was done according to the 8th AJCC staging criteria.[7]

The total number of positive lymph nodes was divided into four groups as per the study done by Roberts *t al*.[8] The four subgroups are as follows: LN: 0, LN: 1, LN: 2–4, and LN ≥5.

Immunohistochemical (IHC) staining for CD44

The IHC for CD44 was done on sections from the tumor proper for all the cases. IHC was performed using the peroxidase and antiperoxidase methods. Following the blocking of endogenous peroxidase activity with peroxidase blocking reagent for 20 mins, the sections were incubated at room temperature for 5–10 mins with primary mouse prediluted monoclonal antibody (prediluted, Clone: HCAM/918, PathnSitu, California) for 1 hour at room temperature. The slides were then rinsed with Tris-buffered saline three times and incubated with Secondary Reagent 2: a conjugated goat anti-mouse polymer horseradish peroxidase (HRP) secondary antibody for 30 mins at room temperature. Diaminobenzidine (DAB) was applied for 5 mins. Hematoxylin was used for counterstaining. Every batch of immunohistochemical staining was tested for specificity using both positive control slides (sections from tonsil) and negative control slides (omitting primary antibody).

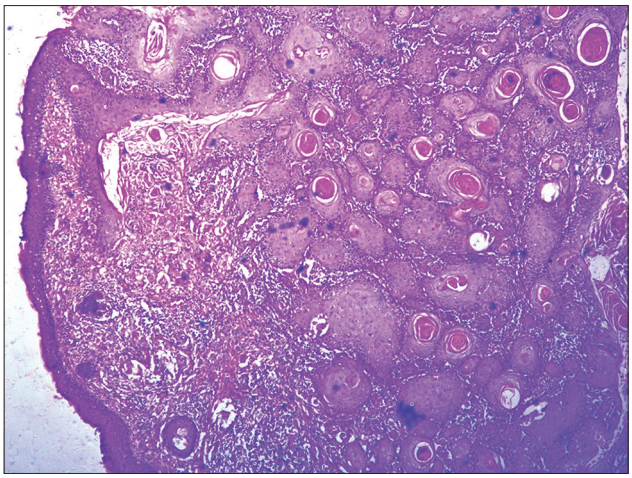

Figure 1: Well-differentiated squamous cell carcinoma (H and E, 40×)

Grading of IHC: two independent pathologists evaluated the expression of CD44. Membranous staining for CD44 was considered positive. The staining of CD44 was interpreted as the product of staining intensity and proportion of the tumor cells. The intensity was graded as 0 = None, 1 = Weak, 2 = Moderate, 3 = Strong. Distribution was graded as 0= <10%, 1 = 11–50%, 2 = 51–80%, 3= >80%.

For the final scoring, the intensity score and the distribution score were multiplied and graded as 0- No expression, 1–4: Low expression, and 5–9: High expression.[9]

Statistical analysis

After entering data into Microsoft Excel, data analysis was done with Statistical Package for the Social Science (SPSS) 22 version software. Frequencies and proportions were used to represent categorical data. As a test of significance, for qualitative data, Chi-square test and Fischer's exact test (for 2 × 2 tables only) were used. *P*-value (probability that the result is true) of <0.05 was considered statistically significant after assuming all the rules of statistical tests. For the assessment of histopathological parameters as independent predictors of recurrence, Cox Regression analysis was used. Statistical software such as

MS Excel and SPSS version 22 (IBM SPSS Statistics, Somers NY, USA) was used to analyze data.

RESULTS

The study population comprised 105 patients of OSCC. The peak incidence was noted in the fourth decade of life (30.4%) with a mean age of 52.59 years. The Male: Female ratio was 1:3.76. This study group was further classified based on gender, grade of tumor, depth of invasion, nodal status, and TNM staging [Table 1]. Low expression of CD44 [Figure 2] was seen in 49 (46.60%) cases, high expression [Figure 3] in 43 (40.9%) cases, and 13 (12.50%) cases showed no expression. A statistically significant association was observed between the expression of CD44 and the depth of invasion, nodal status and TNM stage of the tumor. However, there was no significant association observed between CD44 expression and the histopathological grade of the tumor [Table 2].

A follow-up for a period of 6 months was possible in 70 cases for recurrence of which 18 cases had local recurrence. On comparing the histopathological parameters, the nodal status (number of positive lymph nodes) showed a statistically significant *P*-value ($P = 0.019$). The hazard ratio calculated was 3.457 with 1.227 as the lower limit of the 95% confidence interval.

The majority of the cases (46.60%) showed low expression of CD44 [Figure 2] with 40.90% of cases showing high expression [Figure 3] and 12.50% showing no expression of CD44.

DISCUSSION

CSCs are a subset of cells in the tumors harboring the innate features of stem cells with tumorigenicity. The distinguishing features between CSCs and other stem cells are the altered genetic expressions and the symmetry of their cell division.[10] CSCs define the stemness of the tumors, they not only help in uncontrolled proliferation but are also responsible for the metastatic behavior and development of treatment resistance often resulting in relapse.[11]

Studies evaluating the expression of CD44 in OSCC have shown the frequency of low expression being between 36.4% and 43.29%,[12-14] which is close to the frequency noted in our study (46.6%). A high expression of CD44 was observed in 40.9% of the cases while the other studies have noted a frequency ranging between 20.1% and 33.69%.

The present study has evaluated the association of CD44 expression with the presence of nodal involvement. We observed a statistically significant association ($P < 0.001$) between the high

Table 1: Distribution of patients based on histopathological parameters

Parameters	Frequency (percentage)
Gender	
Male	22 (21%)
Female	83 (79%)
Tumor Grade*	
WDSCC	80 (76.1%)
MDSCC	23 (22%)
PDSCC	2 (1.9%)
DOI†	
0-5	17 (16.2%)
6-10	39 (37.2%)
>10	49 (46.6%)
Nodal Status	
N_+	52 (49%)
N_0	53 (51%)
Positive lymph nodes‡	
LN: 0	53 (50.4%)
LN: 1	21 (20%)
LN: 2-4	22 (20.9%)
LN: ≥5	9 (8.7%)
TNM Staging	
I and II	14 (13.3%)
III	47 (44.7%)
IV	44 (42%)

*WDSCC=Well-differentiated squamous cell carcinoma, MDSCC=Moderately differentiated squamous cell carcinoma, PDSCC=Poorly differentiated squamous cell carcinoma, †DOI=Depth of invasion, ‡LN=Lymph node

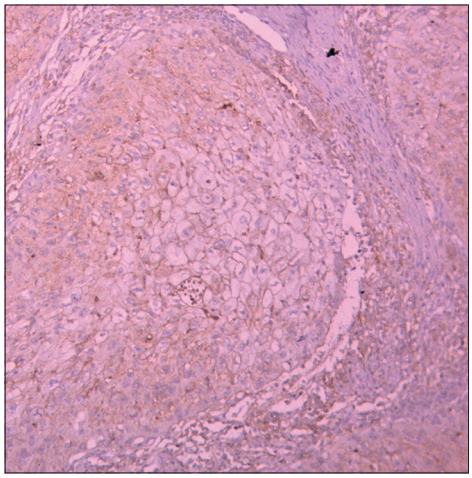

Figure 2: Low expression of CD44 (IHC, 100×)

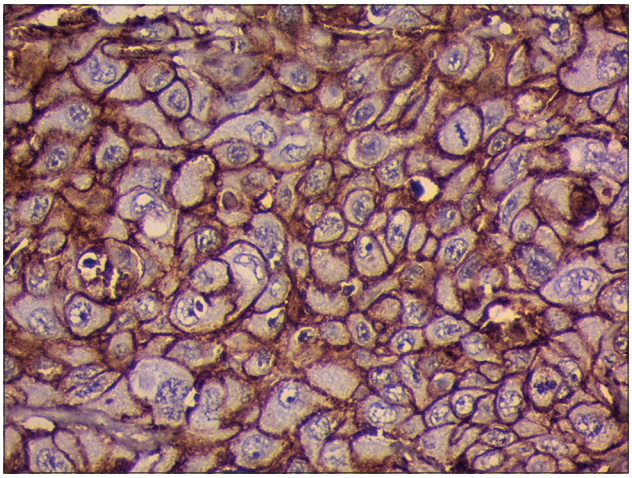

Figure 3: High expression of CD44 (IHC, 400×)

Table 2: Association of CD44 expression with histopathological parameters

Parameter		No expression (13)	Low expression (49)	High expression (43)	*P* value
Grade*	WDSCC (80)	11 (13.7%)	38 (47.5%)	31 (38.8%)	*P*=0.40
	MDSCC (23)	2 (8.6%)	9 (39.1%)	12 (52.3%)	
	PDSCC (2)	0	2(100%)	0	
Nodal Status	N+ (52)	1 (2%)	18 (34.6%)	33 (63.4%)	*P*=0.001
	N0 (53)	12 (22.6%)	31 (58.4%)	10 (19%)	
No. of positive	LN: 0 (53)	12 (22.6%)	31 (58.4%)	10 (19%)	*P*=0.001
lymphnnodes‡	LN: 1 (21)	1 (4.7%)	8 (38%)	12 (57.3%)	
	LN: 2-4 (22)	0	8 (36%)	14 (64%)	
	LN: ≥5 (9)	0	2 (22.2%)	7 (77.8%)	
TNM Stage	Stage I & II (14)	3 (21.4%)	10 (71.4%)	1 (7.2%)	*P*=0.037
	Stage III (47)	6 (12.7%)	23 (49%)	18 (38.3%)	
	Stage IV (44)	4 (9%)	16 (36.3%)	24 (54.7%)	
DOI†	0-5 (17)	8 (47%)	5 (29.4%)	4 (23.6%)	*P*=0.001
	6-10 (39)	3 (7.6%)	14 (35.8%)	22 (56.4%)	
	>10 (49)	2 (4.1%)	30 (61.3%)	17 (34.6%)	

*WDSCC=Well differentiated squamous cell carcinoma, MDSCC=Moderately differentiated squamous cell carcinoma, PDSCC=Poorly differentiated squamous cell carcinoma. ‡LN=Lymph node, †DOI: Depth of invasion

Table 3: Comparison of association of CD44 with lymph node metastasis in different studies

Name of the author	Total no. of cases	High Expression	Low Expression	*P* value
Ortiz RC *et al*.[15]	50	38	12	*P*=0.0181
		N_+:23	N_+:2	
		N_0:15	N_0:10	
Hendawy H *et al*.[16]	44	26	18	*P*<0.05
		N_+:23	N_+:3	
		N_0:3	N_0:15	
Ma C *et al*.[9]	101	68	33	*P*=0.001
		N_+:31	N_+:4	
		N_0:37	N_0:29	
Present study	105	49	43	*P*=0.001
		N_+:18	N_+:33	
		N_0:31	N_0:10	

expression of CD44 and the presence of lymph node metastases. Similar findings have been encountered by other authors like Ortiz *t al*.,[15] Hendawy H *et al*.,[16] and Ma *et al*.[9] in their study population of 50, 44, and 101 patients, respectively [Table 3]. In a study conducted by Roberts TJ *et al*[8] where they have compared different lymph node parameters like the total number of positive nodes, lymph node ratio and the AJCC N stage with the survival of the patients, they have observed that the total number of positive nodes is a better prognostic indicator in comparison to the other parameters. So in this study the expression of CD44 was compared with the total number of lymph nodes positive in which we observed that with increasing number of positive nodes, the frequency of cases showing high expression of CD44 also increased. This signifies the role of CD44 in enhancing the metastatic potential of the tumor.

The current AJCC staging has incorporated the depth of invasion as a separate parameter along with the maximum tumor dimension for the T staging of tumors.[8] This shows that the depth of invasion is an independent indicator of the neoplastic behavior of the tumor. On comparing the expression of CD44 with the depth of invasion, we observed that with increasing depth of invasion, the expression of CD44 also increased showing a statistically significant association between high expression of CD44 and an increased depth of invasion. From this, we can infer that CD44 plays a role in contributing to the neoplastic ability of the tumor by enhancing its invasive potential and aggressive behavior.

The expression of CD44 when compared in the different stages of OSCC, we observed that with advancement in the stage, the expression of CD44 significantly increased ($P = 0.037$). Studies conducted by Ma *et al*.,[5] Hendawy H *et al*.,[16] and Saghravanian *et al*.[13] showed similar observations wherein with increasing stage, the expression of CD44 also increased [Table 4].

Patients with advanced stages of oral cancer continue to have dismal outcomes despite improvements in surgical methods, adjuvant treatment, and understanding of the molecular processes of disease as the current therapies target rapidly proliferating cells and reduce the tumor's size, leaving behind a specific group of tumor cells known as cancer stem cells.[17] These CSCs have the capacity to self-renew, differentiate and are extremely resistant to cytotoxic medicines, often being responsible for metastasis and tumor recurrence.[18] Therefore, realizing the significance of CSCs as potential biomarkers and therapeutic targets, as well as the significance of their identification and characterization essential and identifying their markers, can serve as a method for determining CSCs to target.

Table 4: Comparison of association of CD44 with lymph node metastasis in different studies

Study Conducted	Total Cases	Stage I	Stage II	Stage III	Stage IV	*P* value
Saghravanian *et al*.[13]	45	10 High:1 Low:7	9 High:1 Low:6	13 High:7 Low:5	13 High:2 Low:9	*P*=0.043
Ma C *et al*.[5]	101	I+II = 59 High: 33 Low: 26		III+IV= 42 High: 33 Low: 9		*P*=0.018
Hendawy H *et al*.[16]	44	I+II = 18 High: 2 Low: 16		III+IV = 26 High: 24 Low: 2		*P*=0.000
Present Study	105	I+II = 14 High: 1 Low:10		47 High:18 Low:23	44 High:24 Low:16	*P*=0.037

CONCLUSION

The positive association between high expression of CD44 and lymph node metastasis and pathological TNM staging demonstrates CD44 as a potential biomarker to predict the neoplastic behavior of the tumor.

Biomarker predicting the behavior of the tumor can play a significant role in personalized treatment planning.

Limitations

The present study has two major limitations. It is a unicentric study and although along with CD44, there are other recognized CSC markers in solid malignancies including OSCC, this study aimed at evaluating only CD44 as a single CSC marker.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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