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IDENTIFICATION OF CANCER STEM CELLS THROUGH IMMUNOHISTOCHEMICAL ANALYSIS OF CD133 AND CD44 IN ORAL SQUAMOUS CELL CARCINOMA (OSCC)

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ABSTRACT

Oral cancer is a global public health concern emerging as the sixth most common cancer worldwide. 95% of malignancy found in tumors of oral cavity account for squamous cell carcinoma (SCC). Heavy tobacco consumption, alcohol use, poor nutritional habits, viral infections and various other etiologies implicate to the carcinogenesis of SCC of tongue. This study aims to assess the Immunohistochemical expression of CD44 and CD133 in the different grades of OSCC and to evaluate its role in signaling pathways for cancer progression. The study was conducted in accordance with the Medical Research and Ethical Committee of the Era's Lucknow Medical College and Hospital, Lucknow with the enrollment of 40 subjects clinically diagnosed as OSCC. Immunohistochemical analysis was performed against CD133 and CD44 proteins. Statistical analyses have been done using the IBM-Statistical Package for Social Sciences (SPSS) version 16. Significant correlation was observed with the clinical stages of OSCC and non-significant correlation was seen with demographical parameters. Statistical analysis showed a significant correlation between clinical stages and immunostaining of CD133 marker Strong staining was considered as higher expressions of CD44 and $p<0.05$ was considered significant. Association of CD44 expression and clinicopathological parameters was found in OSCC patients except that of cellular differentiation.

Key words: Squamous Cell Carcinoma (SCC), Cancer Stem Cells (CSC), CD44, CD133

INTRODUCTION

Mouth cancer is a global public health concern emerging as world's sixth most prevalent cancer **[1].** More than 200,000 new patients are detected globally in every year **[2].** It is reported that approximately 30% of global cancer burden is contributed by oral cancer **[3].** 95%of the malignancy contained in oral cavity tumors compensate for squamous cell carcinoma (SCC) **[4].** Through several analyses, it is confirmed that it is more common in males and hence it is ranked eight most prevalent cancers in this category as compared to females where it emerges fifth most common malignancy worldwide **[5].** Heavy tobacco consumption, alcohol use, poor nutritional habits, viral infections and various other etiologies implicate to the carcinogenesis of SCC of tongue. Up to 75% of SCC is attributed to tobacco and alcohol consumption.

Recent studies have demonstrated cancer stem cells (CSCs) are malignant neoplastic cells, which have capability of self-renewal and are termed as tumor initiating cells (TIC). The three common characteristics that aid in identification of CSCs are: (i) Differentiation that enhances its ability to generate heterogeneous progeny, (ii) Self renewal capacity that retains stem cell pool for expansion, (iii) Homeostatic control that ensures tissue specificity of CSCs.

The carcinogenic progression of the disease could be easily visualized along with its initiating clinical diagnostic properties, despite this fact; it is usually detected in its advanced stages **[6].** The process of carcinogenesis progresses with the genetic alterations and mutations in signal transduction pathways that administer various cellular physiological functions such as cell division, differentiation adhesion and apoptosis **[7].** These modifications enhance the ability of a cell to proliferate and metastasize to distant surroundings as compared to other normal epithelial cells **[8].** These various stages of alterations are correlated to the incidence of the disease in terms of histological grading and further prognosis **[9].**Although various surgical and adjuvant therapies have been developed for diagnosis of oral cancer, its prognosis with OSCC remains poor.

CD44 is a cluster of a single transmembrane glycoprotein involved in cellular interactions via hyaluronan, extracellular matrix proteins, and several growth factors **[11].**

It is found up regulated in subpopulations of majority of cancer stem cells (CSCs) and hence recognized as a molecular marker for CSCs. Dalchau et al was the first to illustrate CD44 as a brain granulocyte-T-lymphocyte antigen **[12].** CD44 constitutes a cytoplasmic domain and a transmembrane domain. It is encoded by 20 exons including 10 exons constant in all isoforms and 10 exons result inthe generation of a variable region.

CD133 is 865 amino acid pentaspan transmembrane glycoprotein with a minimum molecular weight of 120 kDa and is concerned with progenitor/stem cells, tumour, growth, differentiation and metabolism. It is located on protrusions of microvilli and other plasma membranes. Its expression is regulated by various extracellular or intracellular factors, and reflects cell form modifications with different functions. It is found in hematopoietic stem cells, glioblastomas, neuronal, glial stem cells, and progenitor endothelial cells, among a few other forms of cells.The invasive mechanism of OSCC requires migration of free cell through extracellular matrix via disruption of intercellular adhesions **[13].**The malignancy advances with structural and functional alterations in CD44 and CD133 which further aids to the detachment of the cell from the site of origin and promotes tumor growth. Hence, CD44 and CD133 requires prominent approach regarding its role in cancer cells in terms of adhesive, locomotive, and growth-transducing functions **[14].**

The increased prevalence of the disease fetches the interest of the pathologists towards the various perspectives of tumor biology focusing on the expression of adhesion molecules. The alterations noticed in their properties gears the invasive mechanism of cancer and its distant metastasis. The loss of cell compaction due to weakened cell-cell adhesion forces harness motility to these cells and allow these malignant cells to migrate distantly from the site of origin through degraded extracellular matrix **[10].**The field of cancer research anchors the clinicopathological impacts of CD44 and CD133 as a molecular target for cancer therapy. This study aims to assess the immunohistochemical expression of CD44 and CD133 in the different grades of OSCC and to evaluate its role in signaling pathways for cancer progression.

Material Methods

Research subjects

The study was conducted in accordance with the Medical Research and Ethical Committee of the Era's Lucknow Medical College and Hospital, Lucknow with the enrollment of 40 subjects clinically diagnosed as OSCC. Both the cases recruited for this prospective study were selected from the Out Patient Department of Department of Surgical Oncology King George's Medical University (KGMU), Lucknow, India. The clinical and demographical records of each patient were obtained from hospital records. Written informed consents were taken from the patients prior to participation in the study.

Inclusion Criteria

(i) Subjects clinically diagnosed for OSCC.

(ii) Subjects who have not received any previous treatment regarding the disease.

Exclusion Criteria

(i) Subjects with other deformities which could affect the analytic outcome of the study were excluded.

(ii) Subjects with AIDS or any other immunodeficiency disorder.

Immunohistochemical analysis

Immunohistochemical analysis was performed against CD133 and CD44 proteins. Formalin fixed, paraffin wax embedded (FFPE) tissues from 40 biopsy specimens were used for our present study. The 3-4 µm thick histological sections were mounted on chemically coated glass slides for CD133 and CD44, deparaffinized in xylene, rehydrated in gradient alcohol (100%, 70%, and 50%) and distilled water. Further blockage of endogenous peroxidase activity is done using3% hydrogen peroxide in methanol for 30 minutes. Antigen retrieval of the specimens was conducted by at high pH solution (DAKO, Denmark) and heated at sub boiling temperature by immersing the tissues in a household streamer after which they are cooled for 15-20 minute.

Buffer was using Phosphate-buffered-saline (PBS) solution (DAKO, Denmark) is done thrice for at least three minutes each at room temperature. Then the tissues were incubated with CD133 primary antibody (polyclonal, 1:50 dilution, Proteintech, USA) and CD44 (monoclonal, 1:50 dilution, Proteintech, USA) for 1.5 hours at room temperature and washed three times by wash buffer. Tissues were then kept in polymer Horse Radish Peroxidase (HRP) for 30 minutes at room temperature and again washed in PBS thrice. Then, DAB (diaminobenzidine) brown chromogen was added to the tissues followed by their washing 5-10 times with water. Counterstaining of tissues was done using Hematoxylin counter stain for 3–5 min at room temperature. After this step the tissues were rinsed in water. The sections were mounted using dibutylphthalate polystyrene xylene and examined under microscope (Leica, Germany).

Statistical analysis

Statistical analysis done using the IBM Statistical Package for Social Sciences (SPSS) version 16. All the Data was presented as the mean ± standard deviation (SD). Comparisons between the cases and controls were analyzed by Chi-Square test/Fisher's exact test using Kruskal–Wallis (H) analysis of variance as appropriate. A two-tailed Probability (p) value $P < 0.05$ was considered statistically significant.

Results

The role of CD133 and CD44 was investigated in human oral carcinogenesis and their expression was evaluated by immunohistochemical staining. A total of 40 patients with OSCC consisting of 22 males (55%) and 18 females (45%) were included in this study. Patient age was categorized into two groups in which higher percentage (655%) was observed in old age group. The topographical distribution of the site of OSCC showed maximum occurrence in the tongue area 24 (55%). Significant correlation was observed with the clinical stages of OSCC **[Table 1]** and non-significant correlation was seen with demographical parameters **[Table 2].** Statistical analysis showed a significant correlation between clinical stages and immunostaining of CD133 marker **[Table 3].**

Higher percentage of individuals with betel nut chewing (77.5%), alcohol consumption (37.5, smoking (35%) was observed in OSCC group as compared to controls, thus they can be highly correlated with increased risk of oral cancer **[Table 4].**

Expression levels of CD44 were categorized as weak expression and strong expressions according to staining intensity. Strong staining was considered as higher expressions of CD44 and $p<0.05$ was considered significant. No association of CD44 expression and clinicopathological parameters was found in OSCC patients except that of cellular differentiation. Greater expression intensities were found in high grade OSCC (moderate and poor differentiation) than in low grade tumors (well differentiated) but this finding was not statistically significant. Similarly increased expression of CD44 was seen in late stage tumors but this difference was also not statistically significant **[Table 5].**

Table 1: Summary of clinicopathological features of OSCC patients

Clinicopathological characteristics	**No. of patients (%)**
Gender	
Male	22(55%)
Female	18(45%)
Age	
<50	14 (35%)
>50	26(65%)
Topography	
Tongue	24(55%)
Oral floor	18(45%)
Other localizations	08(20%)
T- stage	
T1	12(55%)
T2	14(35%)
T3	15(37.5%)
T4	09(22.5%)
Lymph node involvement	
N0	28(55%)
N1	08(45%)
N2	04(10%)
Histological grade	
Well differentiated	12(55%)
Moderately differentiated	8(45%)
Poorly differentiated	6(15%)
Hyperplasia	24(60%)
Associated lesions	
mild	12(30%)
moderate	17(42.5%)
severe	11(55%)
normal epithelium	10(45%)

OSCC: Oral squamous cell carcinoma

Table 2: The distributions of demographical characteristics of OSCC patients

Demographical	No. of OSCC cases (%)
Tobacco Chewers	
Yes	34(85%)
No	6(15%)
Smoking	
Yes	28(70%)
No	12 (30%)
Pan Masala	
Yes	17(42.5%)
No	23(57.5%)
Alcohol	
Yes	5(12.5%)
No	35(87.5%)

OSCC: Oral squamous cell carcinoma

Table 3: Correlation of Immunohistochemical expression of CD133 with clinicopathological parameters of OSCC cases

Clinicopathological characteristics	No. of patients (%)	CD133 positive (%)	p
Gender			
Male	22(55%)	17(42.5%)	0.5
Female	18(45%)	5(12.5%)	52
T- stage			
<2	2(5%)	1(2.5%)	0.3
2-4	12(30%)	7 (17.5%)	54
>4	26(65%)	22(55%)	
Lymph node involvement	35(87.5%)	27(67.5%)	0.4
Yes	5(12.5%)	3(7.5%)	28
No			
Clinical stage			
I-II	23(57.5%)	18(45%)	0.0
III-IV	17(42.5%)	12(30%)	45
Nodal status			
N0	25	21(52.5%)	0.2
N1-N2	(62.5%)	09(22.5%)	31
	5(37.5%)		
Metastatic status			
M0	28(70%)	17(42.5%)	0.1
M1	12(30%)	09(22.5%)	17
Pathological grade			
WD	14(35%)	12(30%)	0.3
MD-PD	26(65%)	20(50%)	14

WD: Well differentiated

MD: Moderately differentiated

PD: Poorly differentiated

Table4: Correlation of Immunohistochemical expression of CD133 with demographical parameters of OSCC cases

Demo graphical	No. of OSCC cases (%)	CD 133 positive	P value
Tobacco Chewers			
Yes	34(85%)	31(77.5%)	0.714
No	6(15%)	2(5%)	
Smoking			
Yes	28(70%)	14(35%)	0.320
No	12 (30%)	12(30%)	
Pan Masala			
Yes	17(42.5%)	14(35%)	0.347
No	23(57.5%)	9(22.5%)	
Alcohol			
Yes	5(12.5%)	15(37.5%)	0.813
No	35(87.5%)	1(2.5%)	

OSCC: Oral squamous cell carcinoma

Table5: Relationship between CD44 expression and clinicopathological parameters

Clinico pathological characteristics	Low Expression	high expression	p
Gender			
Male	26(65%)	17(42.5%)	0.552
Female	18(45%)	5(12.5%)	
T- stage			
<2	2(5%)	1(2.5%)	0.354
2-4	12(30%)	7 (17.5%)	
>4	26(65%)	22(55%)	
Lymph node involvement	35(87.5%)	27(67.5%)	0.428
Yes	5(12.5%)	3(7.5%)	
No			
Clinical stage			
I-II	23(57.5%)	18(45%)	0.045
III-IV	17(42.5%)	12(30%)	

Nodal status N0 N1-N2	25 (62.5%) 5(37.5%)	21(52.5%) 09(22.5%)	0.231
Metastatic status M0 M1	28(70%) 12(30%)	17(42.5%) 09(22.5%)	0.117
Pathological grade WD MD-PD	14(35%) 26(65%)	12(30%) 20(50%)	0.314

WD: Well differentiated

MD: Moderately differentiated

PD: Poorly differentiated

Discussion

India accounts for highest number of oral cancers cases worldwide with approx. 1% of the population reporting for oral premalignant lesions **[15].** In the present study, male OSCC cases are more predominant than females OSCC cases. Through Socio-cultural norms and values of the country male get easy access to availability of tobacco products **[16].** The aggressive marketing of tobacco products not only attracts youths but also children. Tobacco constitutes numerous carcinogens which drags oral cavity which makes it more vulnerable to carcinoma lesions **[17].** Basically, the development of carcinoma is attributed to the contact of tobacco quid in the gingivobuccal sulcus region **[18].** The most common sites for mouth carcinoma are the palate of tongue and mouth floor in western countries due to consumption alcohol consumption and smoking **[19].** It is illustrated that smoking is the foremost mode of tobacco consumption in western countries, while smokeless forms of tobacco consumption are more prevalent in India and Indian subcontinent, including pan masala, khaini, gutkha, etc **[20].** If carcinoma is detected in its early stages its treatment outcomes could be significantly improved **[21].** Due to lack medical amenities, late diagnosis of carcinoma worsens the prognosis of the disease that is a major risk factor in India. Majority of patients were at advanced stage of disease compared to patients with early stages **[22].** The results were consistent with other parts of India they also diagnosed at its advanced stages of carcinoma **[23, 24].** Traditional TNM staging and histopathological grading systems are considered as poor predictors of tumor aggressiveness and do not aid satisfactorily to the subjects **[25].** Therefore, further investigation is needed to explore effective tumor prognostic markers for OSCC patients.

Various cancer stem cell (CSC) have been employed and correlated with certain clincopathological tumor features that serves as a hypothesis for explaining the tumor initiating capacity of tumor cells and their heterogeneity **[26,27,28]**Reliable tumor biomarkers are needed during the pretreatment workup session to envisage long-term prognosis and characterize individual treatment modalities for OSCC patients. The present study focused onto examine the expression of CD133 and CD44 tumor markers via IHC, including stem cell and tumor-related biomarkers, to identify more reliable prognostic markers in OSCC biopsy specimens collected prior to cancer treatment. Recent studies have correlated CD44 high expression levels with OSCC progression.CD44 is highly expressed in poorly differentiated metastatic stages leading to invasive actions, malignant transformations and its poor prognosis.

Studies conducted over OSCC in the last few decades have used various CSCs including CD44 **[29, 30, 31],** CD133 **[32, 33],** CD29 **[34**, **36],** CD117 **[37,35],** and CD97 **[38].**Through these studies it was also revealed that none of them showed high specificity. Data investigated from certain literature showed CD44 as the most commonly used surface marker for identification of OSCC **[39,40,41,42].** CD44 as a surface marker is expressed in both normal oral epithelium (43-47) and in premalignant and malignant lesions **[48–50].** Prince ME et al. suggested that only 10% of CD44+ cells subpopulation oral squamous cell carcinoma initiated new tumours in vivo and possessed a primitive cellular morphology **[10].** CD44 is however found as a poor prognostic marker in case of OSCC. Although, Boxberg M et al observed overexpression of CD44 as a prognostic factor in case of OSCC but its overall its performance was not satisfactory as an independent marker. He found its overexpression in 37% cases in OSCC cases, 16% of lymph node metastases and 39% invasive marginal lesions which was found significantly correlated with poor histopathological differentiation, tumourous activity and transition of epithelial mesenchymal cells via single cell invasion **[51].** Kaza et al suggested through his findings, an altered expression of CD44 with weak immunostaining in poorly differentiated carcinoma cells in OSCC **[52 - 56].** Mostan et. al also postulated reduced expression of CD44 and a significant correlation with cervical LN metastasis.

Regarding CD133 it was recommended as a CSC marker **[53]** and was found to be expressed in a various cancer cells from brain, colon, lung, melanoma and other solid tumors illustrating that these cells have stem cells or progenitor-like properties. In OSCCs, Zhang Q et al only investigated the prospective use of CD133 as a CSC surface marker on tumour specimens **[54].** He also showed that a small population (1–3%) of carcinoma cells were CD133+ with high clonogenicity, invasiveness, and enhanced in vivo tumorigenicity as compared to CD133- cells . Bonetti R et al detected expression of CD133 in the majority of OSCC samples and significantly correlated with stages of tumour and the clinical outcome of patients in terms of disease-free survival **[55, 56].**

Analysis of these two biomarkers enable prognosis of OSCC patients using various clinicalcopathological factors such as tumor, node, metastasis; clinical staging etc. These aspects could assist clinicians for a better prognostic and therapeutic assessment of OSCC patients.

Conclusions:

Both CSC surface markers investigated as indicators found as a reliable marker for outlining CSCs population in solid tumors since they do not portray tumor cells entirely. However, there sensitivity and specificity could be enhanced for the detection of CSCs for which further investigations are desired.

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