

Correlation between Clinical, Pathological Variables and Cancer Stem Cell Markers CD133 and CD44 in Oral Squamous Cell Carcinomas and Oral Submucosal Fibrosis

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Abstract: *Oral Squamous Cell Carcinoma (OSCC) of oral Cavity is a common malignant tumor of the mouth that typically affects elderly men and women. It is more aggressive than conventional squamous cell carcinoma affecting other body regions. The cause of the condition is unknown, but genetic mutations may be involved. Factors that may influence its development include smoking and chewing of tobacco, radiation treatment for other reasons, and exposure to coal tar and arsenic. The squamous cell carcinoma may appear as slow-growing skin lesions. The lesions may ulcerate and cause scarring of the oral cavity. It may be difficult to eat, swallow food, or even to speak. The treatment of choice is a surgical excision with clear margins followed by radiation therapy or chemotherapy, as decided by the healthcare provider. In majority of the cases, the prognosis is good with appropriate treatment. This article overviews the essential points of the correlation between clinicopathological variables and cancer stem cell markers CD133 and CD44 in OSCC patients and OSMF.*

Keywords: CD133, cancer stem cells, CD44, oral squamous cell carcinoma, oral sub mucosal fibrosis, clinical prognostic indicator

1. Introduction

Oral cancer is one of the major health problems throughout the world. By its high incidence and low survival rate, the functional and cosmetic deficiencies that accompany the disease even after the treatment. The occurrence of oral cancer is particularly high among men, for whom it is the eighth most common cancer [1,3]. Half of these new reported cases being diagnosed in advanced stages [4]. It is recognized that solid tumors, including oral squamous cell carcinoma (OSCC), are heterogeneous, due mainly to the ongoing mutations that occur as a consequence of genetic instability and environmental factors [5,6]. Accumulated evidence suggests that cancer stem cells (CSCs) may played an important role in the progression and prognosis of cancers [7]. CSCs, a small subpopulation of cancer cells, possess the ability to initiate neoplasm and sustain tumor self-renewal [8]. One of the challenging problems in identification of CSCs is to find those surface markers that unequivocally characterize such tumor cell population. Most of the markers used until now for this purpose are based on the knowledge obtained from tissue development lineage molecules or are derived from hematopoietic or embryonic stem cells research [9]. Several stem cell markers have been described for HNSCC, such as CD44, Bmi-1, CD133, ALDH1, Nanog, Oct-4 and SOX2 [10, 11, 12]. Since CD133 was identified as a pentaspantransmembrane protein for human hematopoietic stem cells and mouse neuroepithelial cells [13,14,15], many studies have subsequently revealed that CD133 expression is associated with progenitor/stem cells, tumor, regeneration, differentiation, and metabolism. CD133 is one of key biomarkers for isolation and characterization of stem cells. Increasing evidence has shown that CD133 is not only a biomarker, but functions also in cell growth, development and tumor biology. Therefore, in this review, we will summarize the new

functions of CD133. CD133, also called Prominin-1, is a product of a single-copy gene on chromosome 4 (4p15.33) in human or chromosome 5 (5b3) in mice. Human CD133 is a transmembrane glycoprotein of 865 amino acids with a total molecular weight of 120 kDa. This protein consists of an N-terminal extracellular domain, five transmembrane domains with two large extracellular loops, and a 59 amino acids cytoplasmic tail [16]. It is selectively localized in microvilli and other plasma membrane protrusions [17,18]. In general, CD133 positive and CD133 negative cells display different characters. For example, 1) CD133+ and CD133- glioma cells belong to independent cancer stem cell populations; 2) CD133+ glioma cells are derived from primordial CD133- CSCs; 3) CD133- CSCs retain their stem-like features as well as tumor initiation capacity, and can re-acquire CD133 expression in vivo; and 4) Both CD133+ and CD133- CSCs have different expression profiles in transcriptional activities and extracellular matrix molecules [19,20]. The transmembrane glycoprotein of the CD44 family are the major human cell surface receptors for hyaluronate, which also bind extracellular matrix proteins and certain growth factors, and act in a diverse range of physiological and pathological processes such as cellular adhesion, migration, angiogenesis, certain lymphocyte functions, and in the dissemination of malignant cells [22-27]. The CD44 gene is unique for all the various isoforms of the protein, and includes at least 19 exons. Exons 1-5 and 16-19 are spliced together to form a transcript known as CD44s isoform, whereas exons 6-15 are variable, generating several isoforms of the CD44 molecule [28]. Thus, the CD44 family consists of a standard form of CD44 (CD44s) and an alternative splice variant (CD44v). The association of the CD44 family with metastasis formation and prognosis in several tumors has been controversial, particularly in oral cancer, and remains inconclusive [29]. Recent studies have demonstrated that malignant neoplasms are organized as

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hierarchical tissues containing differentiated cells with a small subpopulation of proliferating and undifferentiated cells, the so-called cancer stem cells (CSCs) [30]. The CSC theory postulates that only a specific subset of cancer cells within a tumor exhibits stem cell characteristics (e.g., the ability to self-renew and to proliferate extensively), which can sustain growth and promote the recurrence and metastasis of malignant neoplasms [31,32]. In head and neck squamous cell carcinomas (HNSCCs), the CSCs have been previously observed to occur in low percentages, and it has been demonstrated that they can be characterized according to CD44 expression levels [31,33].

2. Methodology

Sample collection and grading:

Tissue specimens were obtained from 20 patients who have reported with some oral (mouth) problem, like mouth opening, stiffness, burning sensation. Clinically diagnosed case of 20 oral sub mucosal fibrosis, 10 oral carcinoma recruited from patients at OPD department of dentistry of Era's Lucknow medical college and hospital, oral biopsy tissue were collected in formalin from department of surgical oncology, king George medical university Lucknow India.

All the tissues samples were collected under informed consent after a clinical oral examination and confirmed the negative oral infections some of OSMF as well as some of OSCC.

Written consent was obtained from each patient history and their present condition and the previous sign or symptoms of oral infection, it was approved by the Research Ethics Committee. All the patients had a history with use of tobacco, pan masala, cigarette, and smoking intake. Confirmation of all cases OSMF and OSCC were done by Histopathology. The histopathological diagnosis was carried out by experienced pathologists of Era's Lucknow medical college and hospital, have confirmed the histological diagnosis of each lesion.

Immunohistochemistry

CSCs will be investigated in tissue biopsy by Immunohistochemistry at histopathological level by commercially available markers (DAKO), for CD. Immunohistochemical analyses were performed on routinely processed, formalin-fixed, paraffin-embedded tissues employing an avidin-biotin complex immunoperoxidase technique (kit name), as previously described [13-15]. Successive tissue specimens were cut into sections of thickness 5 μ m on chemical coated slides for CD44 followed by de-waxination procedure in xylene and rehydrated in decreasing concentration gradient of alcohol (100% , 70%, 50%) and distilled water . Then the slides were treated with 3% H₂O₂ in methanol for 30mins. To block the endogenous peroxidase activity of the tissue antigen retrieval was achieved under high pH solution (DAKO, Denmark) in pressure cooker for few minutes up to one whistle and cooled for 15-20 minutes. The slides were washed thrice in Phosphate Buffer Saline (PBS) solution (DAKO DENMARK) at room temperature. The slides were incubated for 1.5 hours at room

temperature using primary antibody CD133 and CD44 (polyclonal, 1:50, dilution, Protein-tech, USA). And thrice washed with PBS. Further incubation for 30 mins was carried out at room temperature using enzyme linked secondary antibody Horseradish peroxidase (HRP) followed by washing thrice with PBS buffer . Slides were dipped in diaminobenzidinechromogen for 5-10 mins, rinsed with water and counterstained using haematoxylin for 3-5 mins and followed again by water rinsing. The slides were mounted using dibutyl phthalate polystyrene xylene (DPX) and examined under microscope (Leica, Germany) use of positive and negative control enhanced specificity of every batch Immunohistochemical staining.

Working Hypothesis

Consumption of tobacco/pan masala is associated with oral local area irritation and inflammation, which should be associated with expression of cancer stem cell biomarkers labelled by CD44 and CD133 the present study has been planned to investigate about two steps in chain that is rising of inflammation in oral local area due to consumption of pan masala/tobacco and the second being the presence increased number of cancer stem cells in these oral lesions.

Inclusion criteria:

All the patients will be included who has symptoms of pre-oral cancer and cancer

Exclusion criteria:

- 1) Any patient of oral pre cancer and cancer who has been associated with any other malignancy in present or past or who have any immune deficiency syndrome or tuberculosis
- 2) Any other chronic disease.

Histopathology

In this procedure, tissues are dehydrated through a series of graded ethanol baths to displace the water, and then infiltrated with wax. The infiltrated tissues are then embedded in paraffin thin section (3-5 μ m) to wax blocks. Once the tissue is embedded, it is stable for many years. The most commonly used waxes for infiltration are the commercial paraffin waxes. A paraffin wax is usually a mixture of straight chain or n-alkanes with a carbon chain length of between 20 and 40; the wax is a solid at room temperature but melts at temperatures up to about 65°C or 70°C. After collection of tissue sample of 20 samples of oral cancer and 20 samples of oral sub mucosal fibrosis, for fixation of each tissue sample we used wax and formaldehyde for making wax block. Keep the wax block for 2-3 hours it depends on the size of tissue sample. The pathological changes were observed under light microscope under different magnification

Statistical analysis

Statistical analysis was carried out by using chi square test. Data were expressed as mean \pm standard deviation. A value of $p < 0.05$ was considered statically based on TNM stage and grading the group of OSCC and OSMF. Level of CD133 and CD44 in tissue sample compared with two groups OSCC and OSMF based on histopathological grading well differentiated, moderate differentiated and also poor differentiated.

3. Results

To investigate the role of CD133 and CD44 in human oral carcinogenesis, the expression of CD133 and CD44 was evaluated by Immunohistochemical staining. In which the study conducted constituted 40 cases of OSCC patients with 35 (87.5%) males and 05 (12.5%) females.

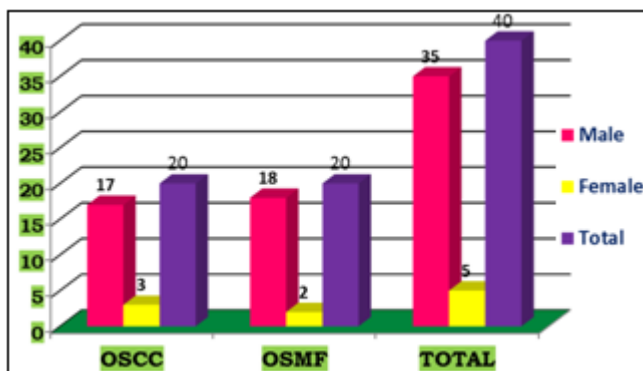
Table I: Categorization of patients according to various demographical parameters such as, tumour size, node involvement, pathological grade, clinical stage, tumour stage in OSCC patients

Tumor size	N (Percent)
>4cm	9 (45.0)
2-4cm	11(55.0)
Node Involvement	
Yes	12 (60)
No	08 (40)
Pathological Grade	
Moderately Differentiated	06 (30)
Well Differentiated	14(70)
Tumor size	
T1-T2	11(55)
T3-T4	09(45)
Lymph Node	
N0	08(40)
N1	10(50)
N2	02(10)

Table II: Non-significant correlation illustrated between males and females and the two categories of OSCC and OSMF ($\chi^2=0.229$; $p=0.633$)

		Group		Total
		OSCC	OSMF	
Sex	Male	17	18	35
		85.00%	90.00%	87.50%
	Female	3	2	5
		15.00%	10.00%	12.50%
Total		20	20	40
		100.00%	100.00%	100.00%

Applied χ^2 test for significance. χ^2 value=0.229; p-value=0.633; consider not significant.



Graph 1: Graphical representation of non-significant correlation illustrated between males and females and the two categories of OSCC and OSMF ($\chi^2=0.229$; $p=0.633$)

Table III: Non-significant and significant correlation of the categories with alcohol consumption ($\chi^2=1.111$; $p=0.292$) smoking ($\chi^2=5.013$; $p=0.025$) and pan masala($\chi^2=12.907$; $p<0.001$).

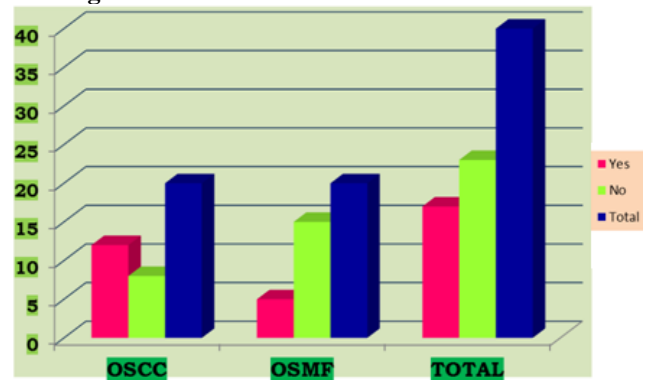
		Group		Total
		OSCC	OSMF	
Smoking	Yes	12 (60.0%)	5 (25.0%)	17 (42.5%)
	No	8 (40.0%)	15 (75.0%)	23 (57.50%)
Panmasala	Yes	7 (35.0%)	18 (90.0%)	25 (62.50%)
	No	13 (65.0%)	2 (10.0%)	15 (37.50%)
Alcohol	Yes	3 (15.0%)	7 (95.0%)	10 (25.0%)
	No	17 (85.0%)	13 (65.0%)	30 (75.0%)

Applied χ^2 test for significance. χ^2 value=1.111; p-value=0.292; consider not significant.

Applied χ^2 test for significance. χ^2 value=5.013; p-value=0.025; consider significant.

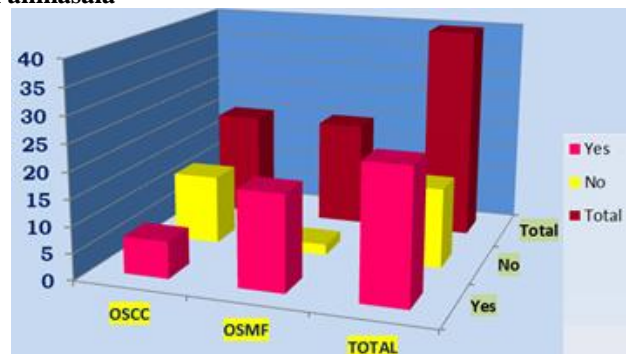
Applied χ^2 test for significance. χ^2 value=12.907; p-value=<0.001; consider highly significant.

Smoking



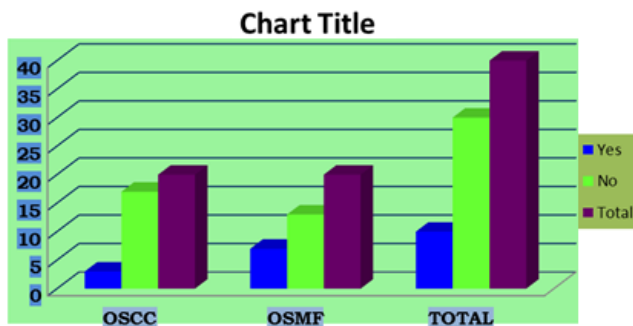
Graph 2: Graphical representation of significant correlation of the category with smoking ($\chi^2=5.013$; $p=0.025$).

Panmasala



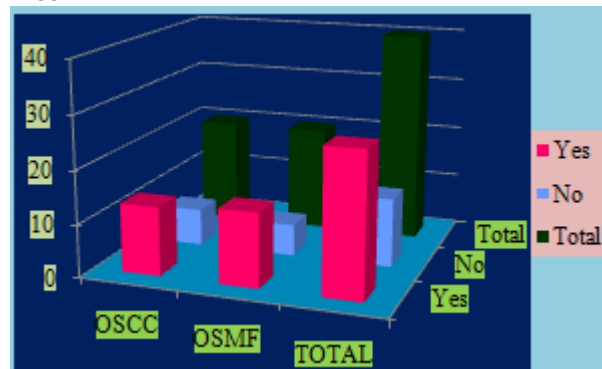
Graph 3: Graphical representation of significant correlation of the category with pan masala($\chi^2=12.907$; $p<0.001$).

Alcohol



Graph 4: Graphical representation of non-significant correlation of the categories with alcohol consumption ($\chi^2=0.114$; $p=0.736$)

CD133



Graph 6: Graphical representation of non-significant correlation in case of tumour marker CD133 with the two categories of OSCC and OSMF consumption ($\chi^2=0.114$; $p=0.736$) and ($\chi^2=0.476$; $p=0.490$)

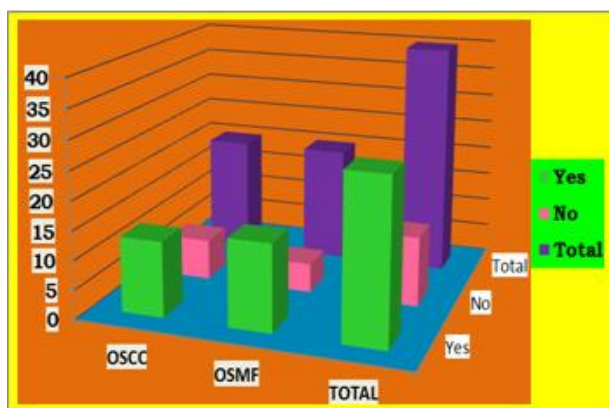
Table IV: A non-significant correlation in case of the two tumour markers CD133 and CD44 with the two categories of OSCC and OSMF consumption ($\chi^2=0.114$; $p=0.736$) and ($\chi^2=0.476$; $p=0.490$)

		Group		Total
		OSCC	OSMF	
CD133	Yes	13	14	27
		65.00%	70.0%	67.50%
CD133	No	7	6	13
		35.00%	30.00%	32.50%
CD44	Yes	13 (65.0%)	15 (75.0%)	28 (70.0%)
	No	07 (435.0%)	05 (25.0%)	12 (30.0%)

Applied χ^2 test for significance. χ^2 value=0.114; p-value=0.736; consider not significant.

Applied χ^2 test for significance. χ^2 value=0.476; p-value=0.490; consider not significant.

CD44



Graph 5: Graphical representation of non-significant correlation in case of tumour marker CD44 with the two categories of OSCC and OSMF consumption ($\chi^2=0.114$; $p=0.736$) and ($\chi^2=0.476$; $p=0.490$)

4. Discussion

The tissue sample was collected and the microscopic study was performed. The tissue was categorized on the basis of tumor size in which 45% tissue are of more than 4 cm, 55% are in the range of 2-4 cm, 60% are showing node involvement, in pathological grading 30% were moderately differentiated and 70% were well differentiated. During the lymph node categorization 40% classified as N0, 50% under N1 and 10% under N2. Non-significant correlation was illustrated in two categories of OSCC and OSMF, in which the males were suffering more than female.

In the case study of smoking, panmasala and alcohol consumers. The passive smokers suffered more as compare to the consumers and in the case of panmasala and alcohol consumers suffered more.

The gene expression level of CD133 and CD44 was also checked.

5. Conclusion

Through this study conducted on 60 OSCC and OSMF patients it was concluded that CD133 and CD44 have correlation with different carcinogen parameters and clinical prognostic indicators. These finding highlights CD44 act as prognostic indicator with respect to clinical stages of OSCC patients, while CD133 are less conclusive. Therefore the advance study are needed to initiate the mechanism and pathogenesis of OSCC and OSMF and the role of CD133 and CD44 prognostic and predictive biomarkers

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