ORIGINAL RESEARCH

Validation of CD44 and CD133 as Prognostic Markers in Oral Potentially Malignant Disorders and Oral Squamous Cell Carcinoma: An Immunohistochemical Study

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Abstract

Introduction: Growing oncogenic research has identified a group of cells within the tumor designated as Cancer Stem Cells (CSC). These CSCs are involved in the genesis, sustained growth, recurrence, and metastasis and in radio resistance of tumors. It is necessary to identify them in order to exploit them as potential therapeutic targets.

Aims And Objectives: To identify the CSCs in Oral Potentially malignant disorders (OPMDs) (leukoplakia and Oral Sub-mucous fibrosis) and Oral Squamous cell carcinoma (OSCC) using markers CD44 and CD133.

Materials and Methods: This ambispective study included 75 cases comprising of, 25 cases of Oral Leukoplakia and 25 cases of OSMF (Oral Sub-mucous fibrosis) –Group-I and 25 cases of OSCC – Group-II, subjected to immunohistochemical analysis using markers CD44 and CD133. Among the 25 cases in each group, ten were prospective cases in each group and fifteen were retrospective cases.

Results: 24 cases of OSCC were positive for CD44 expression and one case was negative. Among Potentially malignant disorders, 33 cases were positive, and 17 cases were negative for CD44 expression. The expression of CD44 differed significantly between OPMDs and OSCC ($p = 0.001$). CD133 expression was absent in all OSCC and OSMF cases, while a small proportion of leukoplakia cases showed positive expression. However, the difference in CD133 expression between the groups was not statistically significant ($p = 0.108$).

Conclusion: The present study demonstrated that CD44 is expressed in OPMDs and OSCC, with cytoplasmic positivity observed in a subset of cells suggestive of cancer stem cell characteristics. These findings support the potential utility of CD44 as a prognostic biomarker and a possible target for future risk stratification and therapeutic approaches. CD133 showed limited utility in the present study and requires further validation as an epithelial stem cell marker.

Key words- CD133, CD44, Cancer stem cell, Oral Submucous fibrosis

Introduction

Squamous cell carcinoma (SCC) is the most common malignant neoplasm of the oral cavity, accounting for about 90% of all oral malignancies.[1] Oral squamous cell carcinoma (OSCC) is a significant cause of morbidity and mortality worldwide, with an incidence of 2%–4% of all cancers.[2,3] In India, oral cancer comprises 40% of all cancers, being the most common in males and third most common in females. Despite advances in diagnosis and therapy, OSCC prognosis remains poor, especially in advanced stages, with high recurrence and mortality rates.[4] Late-stage diagnosis continues to be a major concern.

Numerous studies have confirmed that most OSCCs arise from OPMDs. Delayed recognition of OPMDs contributes significantly to malignant transformation.[5] The World Health Organization (WHO) estimates the annual malignant transformation rate of OPMDs in India at 0.3%. Oral leukoplakia (OL) and oral submucous fibrosis (OSMF) are among the most frequently diagnosed OPMDs, with transformation rates of 17%–35% and 7%–13%, respectively[6]. Therefore, better predictors of malignant transformation are needed.[7]

Recent evidence correlates cancer stem cells (CSCs) with

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the initiation, progression, recurrence, and metastasis of OSCC.[8] CSCs, which constitute less than 1% of tumor populations, possess stem cell-like abilities of self-renewal and differentiation.[8] These cells exhibit features such as active telomerase, resistance to apoptosis, and high migratory capacity, contributing to therapeutic failure and relapse.[9]

The concept of CSCs was first demonstrated in acute myeloid leukemia by Bonnet and Dick in 1997.[10] Subsequent studies and clonogenic assays have supported the presence of CSCs in solid tumors as well.[9] Two main models explain tumor origin: the CSC theory and the clonal evolution (CE) model. The former suggests that a subset of tumor cells with stem-like properties drive cancer growth, while the latter attributes tumor maintenance to genetic and epigenetic variability among all tumor cells.[11]

CSC-related pathways, including Wnt, NF-κB, and Sonic Hedgehog, are crucial for self-renewal, resistance to therapy, and tumor progression.[12] Hence, these pathways are potential therapeutic targets. CSCs and normal stem cells express specific surface markers. Among the most studied CSC markers are CD44 and CD133.[13] CD44 is an 85–90 kDa transmembrane glycoprotein involved in cell adhesion, migration, and interaction with hyaluronic acid. It promotes tumor cell proliferation and survival through MAPK and PI3K/AKT activation.[14] CD44 also plays a role in metastasis in several cancers and is associated with epithelial-mesenchymal transition (EMT)and therapy resistance.[15]

CD133 (Prominin-1) is a ~120 kDa pentaspan transmembrane glycoprotein, originally identified on hematopoietic stem cells. It has since been found in CSCs of brain, liver, lung, gastric, and head and neck cancers.[16] CD133-positive CSCs exhibit chemoresistance and express stemness genes such as Nestin, BMI1, and Nanog[16]. Wei Liu et al. demonstrated the prognostic utility of CD133 and ALDH-1 in oral leukoplakia.[17]

Given the critical role of CSCs in carcinogenesis, it is essential to identify and characterize them using specific mark-

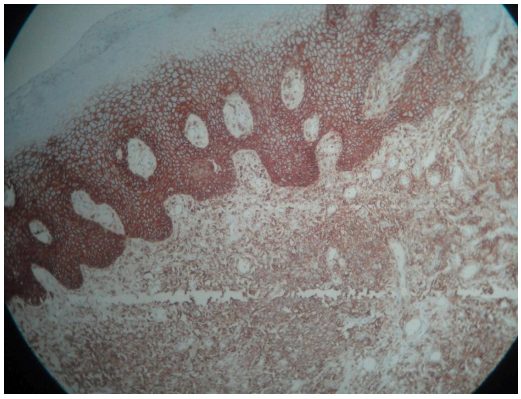

Fig. 1: Photomicrograph showing CD44 positivity in the basal and parabasal cells in Hyperkeratosis of severe dysplasia (CD44 immunohistochemistry, ×100)

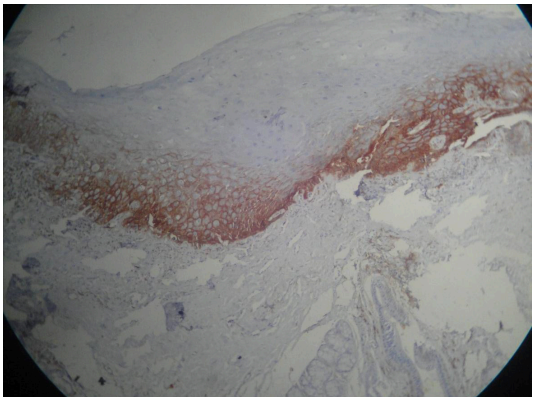

Fig. 2: Photomicrograph showing mild CD44 positivity in the basal and parabasal cells in Oral Sub-mucous fibrosis (CD44 immunohistochemistry, ×100)

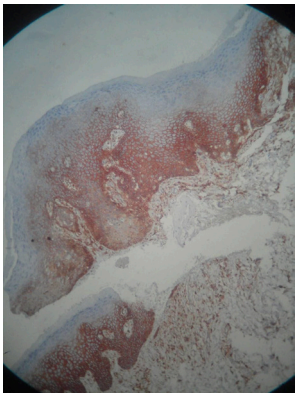

Fig. 3. Photomicrograph showing strong membranous positivity for CD44 in a representative case of OSCC (CD44 immunohistochemistry, ×100)

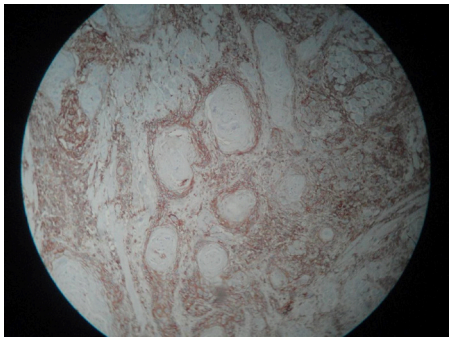

Fig. 4: Photomicrograph showing CD44 positivity in the peripheral tumor cells and negativity in the central areas of keratin pearl formation in OSCC (CD44 immunohistochemistry, ×100)

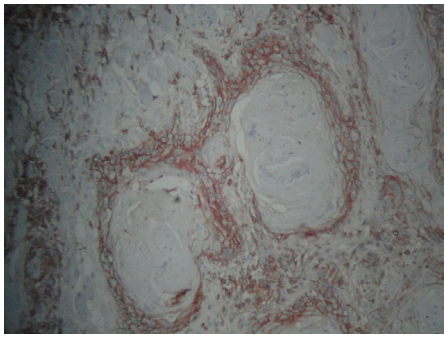

Fig. 5: Photomicrograph showing CD44 positivity in the peripheral tumor cells and negativity in the central areas of keratin pearl formation in OSCC (CD44 immunohistochemistry, ×400)

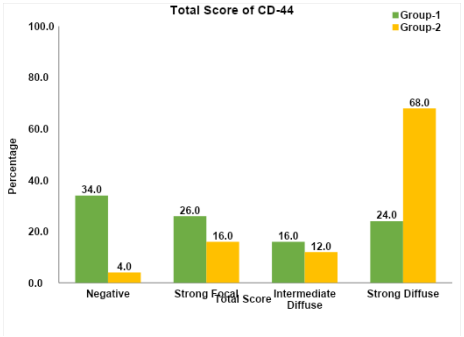

Fig. 6: shows Total Score of CD44

ers. Although several studies have investigated cancer stem cell markers in oral epithelial malignancies, the reported expression patterns of CD44 and CD133 remain inconsistent across studies. Furthermore, comparative evaluation of these markers across the spectrum of oral potentially malignant disorders and established OSCC is limited. Understanding their expression in both premalignant and malignant lesions may provide insights into cancer stem cell involvement during oral carcinogenesis and help identify potential prognostic biomarkers. Hence, this study aims to evaluate the immunohistochemical expression of CD44 and CD133 in OPMDs and OSCC, and assess their role in identifying CSC populations.

Materials and Methods

Sample Selection

This ambispective study comprised 75 samples, divided into two groups: Group I – OPMDs (n = 50) and Group II – OSCC (n = 25). Both retrospective and prospective samples were included. Retrospective cases were retrieved from the archives of the Department of Oral and Maxillofacial Pathology, Saveetha University, from 2010 to 2023. Prospective samples were obtained from biopsied tissues of patients visiting the department between 2021 and 2023, who were under follow-up. The distribution of cases is summarized in Table 1. Inclusion criteria comprised histopathologically confirmed cases of oral leukoplakia with epithelial dysplasia (mild, moderate, or severe), oral submucous fibrosis, and oral squamous cell carcinoma. Both retrospective archival cases and prospective biopsy specimens were included, provided that adequate formalin-fixed paraffin-embedded tissue was available for immunohistochemical analysis and complete clinicopathological data were accessible. Cases with inadequate tissue for immunohistochemical evaluation, poorly preserved or improperly fixed specimens, incomplete clinical records, or those lacking a definitive histopathological diagnosis were excluded from the study.

Diagnosis was confirmed through histopathological evaluation of Hematoxylin and Eosin-stained sections. Epithelial dysplasia was graded using the criteria proposed by Neville et al. OSCC was classified according to the WHO 2017 Classification of Head and Neck Tumours. Oral Submucous Fibrosis was diagnosed based on the criteria described by Pindborg and Sirsat. This study received approval from the Institutional Review Board of Saveetha University (Approval No: SRB/SDMDS130MP3). The study complied with the Declaration of Helsinki.

Immunohistochemistry

Immunohistochemical staining was performed using anti-CD44 antibody (Clone 156-3C11, Thermo Fisher Scientific, USA) and anti-CD133 antibody (GX-7121P, 171–191 aa, Puregene, Genetix Biotech Asia Pvt. Ltd., India). Sections of 3 µm thickness were mounted on gelatin-coated slides, deparaffinized, rehydrated, and subjected to antigen retrieval using citrate buffer (pH 6.0). After blocking endogenous peroxidase and non-specific binding, sections were incubated with primary antibodies at 37°C (CD44 for 60 minutes and CD133 for 120 minutes), followed by application of post-primary block, Novolink polymer (Leica Microsystems, UK), and DAB chromogen. Counterstaining was done with Mayer's hematoxylin, and slides were dehydrated and mounted using DPX. Esophageal and renal cell carcinoma tissues served as positive controls, while omission of the primary antibody was used as a negative control.

Evaluation of Staining

CD44 and CD133 expression was assessed based on staining intensity (negative, mild, moderate, strong) and distribution (negative, focal, diffuse). A composite score was calculated as follows: 0 = negative, 1 = strong focal, 2 = intermediate diffuse, and 3 = strong diffuse. Two independent observers, blinded to the clinical details and diagnostic grouping of the cases, evaluated the slides. Any disagreements were resolved by consensus using a multi-headed microscope.

Sample Size Determination:

The sample size was determined with reference to previously published studies evaluating cancer stem cell markers in OSCC and OPMDs, particularly the study by Mărgăritescu et al.[18] Due to the ambispective nature of the study, the number

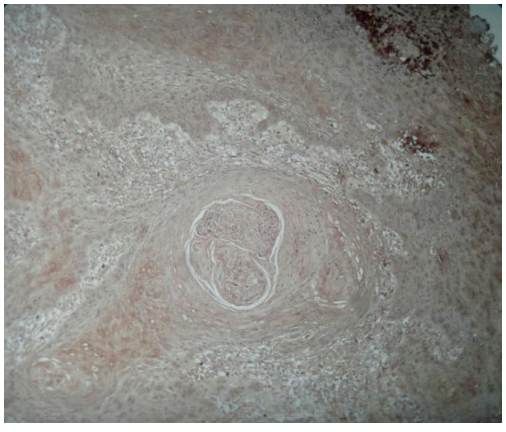

Fig. 7: shows Photomicrograph showing CD133 negativity(CD133 immunohistochemistry, ×100)

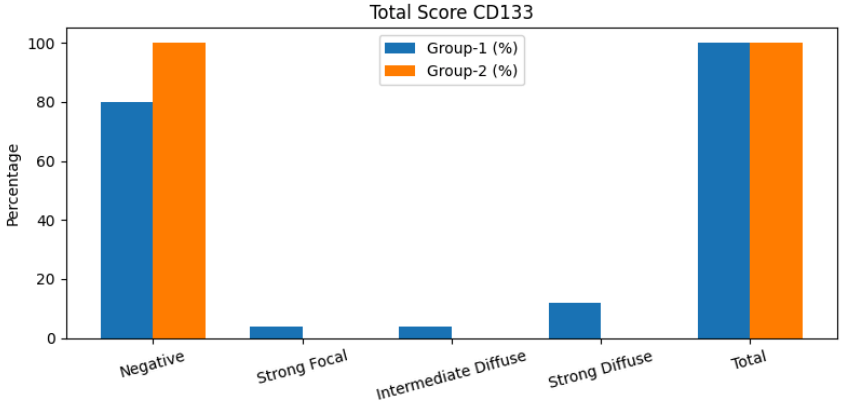

Fig. 8: shows total score of CD133

of cases included was based on the availability of eligible retrospective archival specimens and prospective biopsy samples during the study period. A total of 75 cases satisfying the inclusion criteria were included for analysis. Statistical consultation was obtained to ensure the appropriateness of the sample size and analytical methods employed.

Statistical Analysis

All parameters were tabulated and analyzed using SPSS software version 23.0. The differences in CD44 and CD133 expression patterns between the two groups were assessed using the Chi-square test. Fisher's exact test was applied when the expected cell frequency in any category was less than five. A p-value <0.05 was considered statistically significant.

Table 1: Distribution of cases

Groups	Prospective Cases	Retrospective Cases	Total
Group I Hyperkeratosis with dysplasia (leukoplakia)	10	15	25
Oral Submucous fibrosis	10	15	25
Group II Oral Squamous Cell Carcinoma	10	15	25

Results

A total of 75 samples were included: 50 cases of Oral Potentially Malignant Disorders (OPMDs) and 25 cases of Oral Squamous Cell Carcinoma (OSCC). The OPMDs included 25 cases of leukoplakia (hyperkeratosis with mild, moderate, or severe dysplasia) and 25 cases of Oral Submucous Fibrosis (OSMF) in varying stages (early, moderately advanced, and advanced). Both retrospective and prospective samples were analyzed.

CD44 Expression

Leukoplakia (Group I):

Among prospective leukoplakia cases (n=10), 50% showed strong diffuse CD44 positivity, 10% intermediate, 30% mild focal, and 10% negative. Membranous staining was present in 80%, with 30% showing additional cytoplasmic staining. Most cases (80%) exhibited positivity in both basal and parabasal layers (Figure 1).

In retrospective leukoplakia cases (n=15), 33.3% showed strong diffuse staining, 26.7% mild focal, 20% intermediate, and 20% were negative. Membranous positivity was again seen in 80%, with 10% showing both membranous and cytoplasmic staining (Figure 1)

Table 2 shows Total Score of CD44

Total Score CD44	Group					
	Group-1		Group-2		Total	
	N	%	N	%	N	%
Negative	17	34.0	1	4.0	18	24.0
Strong Focal	13	26.0	4	16.0	17	22.7
Intermediate Diffuse	8	16.0	3	12.0	11	14.7
Strong Diffuse	12	24.0	17	68.0	29	38.7
Total	50	100.0	25	100.0	75	100.0

Chi-Square Test	Value	P-Value
Fisher's Exact Test	15.574	0.001

Table 3 shows total score of CD133

Total Score CD133	Group					
	Group-1		Group-2		Total	
	N	%	N	%	N	%
Negative	40	80	25	100	65	86.67
Strong Focal	2	4	0	.0	2	2.67
Intermediate Diffuse	2	4	0	.0	2	2.67
Strong Diffuse	6	12	0	.0	6	8
Total	50	100.0	25	100.0	75	100.0

Chi-Square Test	Value	P-Value
Fisher's Exact Test	0.0124	0.108

OSMF (Group I):

In prospective OSMF cases (n=10), 50% were negative, 30% focal positive, 10% strong diffuse, and 10% intermediate diffuse. Membranous positivity was noted in 50%, with variable basal/parabasal distribution.

In retrospective cases (n=15), 53.3% were negative, 20% each showed strong focal or intermediate staining, and 6.7% had strong diffuse staining. All positive cases showed membranous staining (Figure 2)

OSCC (Group II):

In prospective OSCC cases (n=10), 60% were strongly positive, 10% intermediate, 20% strong focal, and 10% negative. Membranous staining was present in 80%, with 10% showing both membranous and cytoplasmic positivity (Figure 3).

Retrospective OSCC cases (n=15) showed 100% CD44 positivity: 73.4% strong diffuse, 13.3% strong focal, and 13.3% intermediate. Membranous staining was observed in 93.3%, and 26.7% showed combined membranous and cytoplasmic positivity.

CD44 expression in OSCC was predominantly located at the periphery of tumor nests and islands, while differentiated cells were largely negative (Figure 4 & 5). Chi-square analysis showed a statistically significant difference in CD44 expression between OPMDs and OSCC (p=0.001). The overall distribution of CD44 total scores in OPMDs and OSCC cases is shown in Figure 6, demonstrating a predominance of strong diffuse positivity in OSCC compared to OPMDs.

CD133 Expression

Leukoplakia (Group I):

In prospective cases (n=10), 70% were negative. One case each showed strong diffuse, strong focal, and intermediate diffuse positivity.

In retrospective cases (n=15), 66.7% were negative, while 33.3% showed strong diffuse, strong focal, or intermediate diffuse positivity (Table 3).

OSMF (Group I):

All prospective and retrospective OSMF cases were negative for CD133 expression (Table 3) (Figure 7,8).

OSCC (Group II):

All 25 OSCC cases (prospective and retrospective) were negative for CD133 expression (Table 3) (Figure 7,8).

Overall scoring:

The total CD133 scores in OPMDs and OSCC cases are summarized in Table 3 and Figure 8, demonstrating that most OSMF and OSCC cases are negative, with only a few leukoplakia cases showing focal or diffuse positivity. Fisher's exact test did not show a statistically significant difference in CD133 expression between OPMDs and OSCC (p = 0.108).

Discussion

Oral squamous cell carcinoma (OSCC) is a multifactorial malignancy that progresses through a multistep cascade of genetic and molecular events.[19,20] Despite advancements in research and therapy, the survival rate remains approximately 50%.[21] Increasing evidence supports the CSC theory, which postulates that a subset of long-lived cells such as stem cells undergo cumulative oncogenic insults to give rise to cancer.[22] These CSCs are believed to be responsible not only for tumor initiation but also for recurrence, metastasis, chemoresistance, and radioresistance.[15] Therefore, the identification of CSCs in OSCC is essential for developing effective, targeted therapeutic approaches. However, identifying reliable markers remains a challenge. Among several explored markers ALDH1, ABCG2, Bmi-1, CD44, Oct-4, and CD133, CD44 and CD133 are widely studied in epithelial malignancies, including head and neck squamous cell carcinoma (HNSCC).[23]

In the present study, CD44 and CD133 expressions were evaluated in both retrospective and prospective samples of OPMDs including leukoplakia and oral submucous fibrosis (OSMF) and OSCC. The prospective design included follow-up for malignant transformation in OPMDs and recurrence or metastasis in OSCC, facilitating a better understanding of the continuum of carcinogenesis and the potential role of CSCs. CD44, a cell-surface transmembrane glycoprotein and a hyaluronan receptor, is known to regulate cell adhesion, migration, and signaling.[24] In this study, 68% of OSCC cases showed strong diffuse CD44 positivity, with additional cases demonstrating intermediate or focal positivity. These findings are in accordance with Mack et al.[25], who also reported high CD44 expression in OSCC compared to normal mucosa.

Interestingly, some OSCC cases showed cytoplasmic as well as membranous CD44 staining. This cytoplasmic localization may reflect the proteolytic cleavage of CD44 by ADAM proteases and MMP-14, leading to the formation of an intracellular domain that translocates to the nucleus and activates transcription of genes involved in tumorigenesis.[26–28] This subset of CD44-positive cells with cytoplasmic localization could represent the cancer stem cell population, which typically constitutes less than 10% of the tumor mass[29], with as few as one CSC per 2,500 tumor cells in HNSCC.[30] Additionally, CD44 expression was observed mainly at the periphery of tumor nests and not in the central keratin pearls, indicating its association with proliferative activity and a less differentiated phenotype. This pattern may reflect the hierarchical growth of tumors, resembling the organization of normal stratified epithelium.

In OPMDs, particularly leukoplakia, CD44 was also expressed predominantly in the basal and parabasal layers. About 40% showed strong diffuse positivity, while a few cases exhibited both membranous and cytoplasmic staining. The expression of CD44 in dysplastic epithelium is believed to result from mutations and alternative splicing that generate isoforms contributing to early tumorigenic changes.[31] Some theories suggest that precancerous lesions may harbor stem-like cells that expand through symmetric division and potentially accumulate mutations leading to malignancy.[32] In OSMF, over 50% of cases were negative for CD44, likely due to epithelial atrophy. However, positive cases showed strong membranous staining, which may either indicate normal expression or early aberrant activity. Follow-up of cases with cytoplasmic CD44 localization may provide critical insights into their potential for malignant transformation.

In contrast, CD133 expression was absent in all OSCC and OSMF cases. A small subset of leukoplakia cases demonstrated

CD133 positivity; however, the overall difference in expression between OPMDs and OSCC was not statistically significant. This discrepancy from other studies[17, 33, 18] could stem from differences in antibody clones or epitope masking due to glycosylation, as suggested by Miraglia et al.[34] and Kemper et al.[35]. CD133's glycosylation status can vary with tissue type and differentiation stage, leading to inconsistent detection in immunohistochemistry.[36] Therefore, although CD133 is a known hematopoietic stem cell marker, its utility in identifying epithelial CSCs remains questionable. More robust and reproducible methods, such as PCR or flow cytometry using standardized clones, may improve the detection of CD133 in future studies. Until then, CD44 especially with cytoplasmic localization might remain a more dependable surrogate marker for identifying CSCs in oral epithelial lesions.

The findings of the present ambispective study suggest that CD44 may serve as a useful adjunctive biomarker associated with disease progression and warrants further investigation as a prognostic marker and for recognizing cancer stem cell–like populations in OSCC. The higher expression of CD44 in OSCC compared with OPMDs indicates its possible role in tumor progression. Identification of such cell populations may aid in risk assessment, prognostication, and the development of targeted therapies aimed at eliminating cancer stem cells, thereby potentially reducing recurrence and improving treatment outcomes.

Study Limitations: The present ambispective study was limited by the relatively small sample size and the inclusion of cases from a single institution, which may affect the generalizability of the findings. In addition, the identification of cancer stem cell populations was based solely on immunohistochemical expression of CD44 and CD133 without molecular validation using techniques such as polymerase chain reaction (PCR), flow cytometry, or gene expression analysis. The follow-up period was also limited for assessing malignant transformation in OPMDs and recurrence or metastasis in OSCC. Therefore, larger multicentric studies with long-term follow-up and molecular validation are required to confirm the role of these markers in oral carcinogenesis.

Conclusion:

The present ambispective study demonstrated significantly higher expression of CD44 in OSCC compared with OPMDs, supporting its association with tumor progression and its potential role in identifying cancer stem cell populations. The presence of cytoplasmic CD44 expression in a subset of cases may indicate cells with enhanced stem cell–like properties and malignant potential. In contrast, CD133 expression was not detected consistently, limiting its utility as a reliable marker in the studied lesions. Clinically, CD44 may serve as a useful adjunctive prognostic biomarker for evaluating the progression of OPMDs and OSCC. Further large-scale studies incorporating molecular validation and long-term follow-up are required to confirm these findings and establish their therapeutic implications.

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Consent for publication - This study utilized archived, anonymized formalin-fixed paraffin-embedded (FFPE) tissue blocks from the departmental archives for immunohistochemical analysis. No identifiable patient information was used, and no direct patient contact occurred. Hence, obtaining individual informed consent was not applicable.

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