Original Research

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Immunohistochemical Approach to Evaluate and Compare the Expression of CD44 in Oral Premalignant Disorders and Oral Squamous Cell Carcinoma - A Retrospective Study

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Abstract

Objective: Oral squamous cell carcinoma (OSCC) is the most prevalent kind of head and neck cancer. Oral premalignant diseases (OPMD) such as oral submucous fibrosis (OSMF) and oral lichen planus (OLP), have a higher risk of developing into OSCC. Due to the cellular milieu created by exposure to smoking, alcohol, betel nuts, or the human papillomavirus, the stratified squamous epithelium that covers the oral cavity is extremely sensitive and vulnerable to carcinogenic damage. This can further lead to the formation of dysplastic or hyperkeratotic epithelium, which further transforms to OPMDs. Recently, it has been shown that cancer cells display the cell surface glycoprotein cluster of differentiation 44 (CD44), which may be cleaved at the ectodomain to produce soluble CD44. The aim of the present study is to evaluate and compare CD44 expression as a prognostic marker for OPMD-related cancer development using immunohistochemistry (IHC).

Materials and Methods: The expression of CD44 was assessed by IHC in 80 paraffin-embedded tissues [20 normal mucosa (control), 20 OLP cases, 20 OSMF cases, and 20 OSCC patients] in this retrospective investigation. The one-way analysis of variance (ANOVA) and an independent student's t-test were used to conduct the statistical analysis.

Results: Using an independent sample t-test, comparisons between the control vs OLP, control vs. OSMF, control vs. OSCC, OLP vs. OSCC, and OSMF vs. OSCC all demonstrated statistical significance with p-values of <0.001, <0.001, <0.001, 0.001, and 0.001, respectively. However, a statistical analysis of the comparison between OLP and OSMF with a p-value of 0.894 revealed no statistical significance.

Conclusion: Thus, it has been concluded that CD44 can be employed as a diagnostic marker for the change from healthy mucosa to premalignant and malignant tissue based on the level of expression using IHC.

Keywords: CD44, oral squamous cell carcinoma, oral lichen planus, oral submucous fibrosis, immunohistochemistry

Introduction

The most prevalent kind of head and neck cancers is oral squamous cell carcinoma (OSCC), which is characterized by aggressive malignant characteristics and arises from the squamous epithelium of the oral mucosa (1). OSCCs may be difficult to be diagnosed and treated due to the complexity of the oral tumor microenvironment, where diversity of tumor composition regulates carcinogenesis, malignant growth, as well as therapeutic response (2). Nevertheless, this is true even though the mechanisms underlying have not yet been fully uncovered (3). The stratified squamous epithelium that lines the oral cavity is extremely sensitive and prone to carcinogenic damage (4). The cellular microenvironment caused by exposure to smoke, alcohol, betel nuts or the human papillomavirus can lead to the development of dysplastic or hyperkeratotic epithelium (5). Oral leukoplakia, erythroplakia, lichen planus, and oral submucous fibrosis (OSMF) are some clinical manifestations of this called the oral premalignant disorders (OPMD). Dysplastic epithelium may be observed for any of these disorders, despite the fact that the rate of advancement to cancer differs among distinct situations,

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highlighting the need for histological examination (6). OPMDs make up to 17-35% of all newly diagnosed instances of oral cancer and they progress to cancer on average at a rate of 0.7% to 2.9% each year (7). Different risk factors, many of which are also associated with OSCC, have been linked to these lesions, although it is not yet unknown how these risk factors lead to malignant changes (8). Moreover, diagnostic markers are needed, due to the rising prevalence of OPMDs in order to diagnose it early and stop it from developing into something more malignant. The genesis, development and treatment resistance of cancers are influenced by a number of indicators associated with cancer stem cells (CSCs) that have been discovered in recent years (9). The cell surface glycoprotein cluster of differentiation 44 (CD44), a CSC marker, is crucial for cell motility and adhesion, tumor invasion, cancer prognosis and metastasis (10). Existing publications have demonstrated that metalloproteinase membrane type 1-matrix metalloproteinase may cleave CD44 at the ectodomain, releasing soluble CD44, and that CD44 is expressed in tumor cells (11). It is therefore necessary for the migration of cancerous cells (12).

Investigation of the importance of CD44 expression in oral lichen planus (OLP), OSMF, and oral squamous cell cancer in this paper is presumably the first of its kind. Evaluation of CD44 expression as a prognostic marker for OPMD-related cancer development is the study's primary objective.

Materials and Methods

General Study Details

A retrospective analysis at a single institution was carried out between July 2022 and December 2022. The institution's Oral Pathology Department's archives were used to gather the blocks needed for the investigation. The study received approval from the Institutional Review Board (approval number: SRMU/M&HS/SRMDC/2022/PG/012 - SRM Dental College) on June 29, 2022. As this was a retrospective research, informed consent was not obtained. The study was carried out in accordance with the ethical standards indicated in the Declaration of Helsinki, the Good Clinical Practice guidelines, and the recommendations established by the Indian Council of Medical Research.

Participants

The archives of the institution's oral pathology department yielded paraffin blocks for eighty cases, which included OSCC, OLP, OSMF and normal mucosa (normal mucosa of non-inflammatory hyperplastic epithelial lesions) (each having 20 cases), respectively. Patients' demographic information, such as age and gender, was also obtained from the patient files. The final histopathology report served as the basis for the inclusion criteria. Three pathologists reaffirmed the diagnosis using a compound light microscope. Tissues that were incorrectly or inadequately fixed were excluded. The blocks of the selected cases were further processed for the assessment of CD44 using immunohistochemistry (IHC).

Study Methodology

Immunohistochemistry Procedure for CD44 Staining

Using a microtome, the case blocks were sliced again into the sections of 5 μm thick and were embedded onto coated slides. Slides were rehydrated in ethanol of various proportions after being deparaffinized in xylene. Utilizing a heat retrieval technique while operating under stream pressure, antigen was recovered using a Tris-EDTA buffer. For the optimum epitope recovery, the slides were again transferred to distilled water once the solution had cooled to room temperature. Application of hydrogen peroxide for 10 minutes stopped endogenous peroxidase activity. Using diluted primary mouse antibody, the tissue sections were coated, and they were then let to sit at room temperature for 45 minutes. After being removed with wash buffer, the sections were coated with polythene target binder and let to sit at room temperature for 45 minutes. The slides were then coated in HRP-labelled polymer and let to sit at room temperature for 12 minutes. A brown precipitate formed at the antigen location after 5 minutes of incubation with the substrate-chromogen 3,3'-diaminobenzidine. This completed the staining process. After being twice rinsed with distilled water, the slide was counter stained for 30 seconds before being washed. After drying, the slide was mounted using a synthetic mounting medium. Tonsillar tissue samples were employed as a positive control, while the exclusion of the primary antibody was used as a negative control.

At magnifications of 4x, 10x, 20x, and 40x, all slides were inspected using a compound microscope. For grading the immunostaining pattern, the most significant tumor regions were chosen. An established semi quantitative scoring method with a scale for intensity (I) ranging from one to four was used to assess the degree of CD44s antibody positivity, such as none, mild, moderate, and strong; and for distribution (D), such as none, focal, patchy, and diffuse. Weakly positive tissues were those with I x D of 4 or less, while strongly positive tissues were those with I x D of more than 4 (13).

Statistical Analysis

A total sample size of 80 was calculated using the tool G*Power 3.1.9.4 with the inputs: Effect size f=0.48, α err prob=0.05, Power (1- β err prob)=0.95, and the number

of groups was 4. The data were analyzed in a descriptive and comparative way using the software of Statistical Analysis for Social Sciences (SPSS version 22). The One-Way ANOVA test was used to compare the groups. The independent student's t-test was used to compare the three groups. The result was considered significant at $p<0.05$.

Results

In the current study, 80 cases involving both men and women were examined. Twenty patients each made up four groups: Control, OLP, OSMF and OSCC (Figure 1).

The patients' ages varied from 39 to 71 years (Table 1). Females outnumbered males in all the categories. All

Table 1. Descriptive statistics for CD44 in control, OLP, OSMF and OSCC groups

Groups	Sample size (n)	Age group (years)	Sex		CD44 (I x D)
			Male	Female	Mean ± standard deviation
Control	20	40-70	9	11	2.7 ± 0.8
OLP	20	41-60	4	16	5.4 ± 2.5
OSMF	20	39-64	7	13	5.5 ± 2.1
OSCC	20	45-71	8	12	7.8 ± 1.6
Total	80	39-71	28	52	5.4 ± 2.6

CD44: Cluster of differentiation 44, OLP: Oral lichen planus, OSMF: Oral submucous fibrosis, OSCC: Oral submucous fibrosis, I: Intensity, D: Distribution

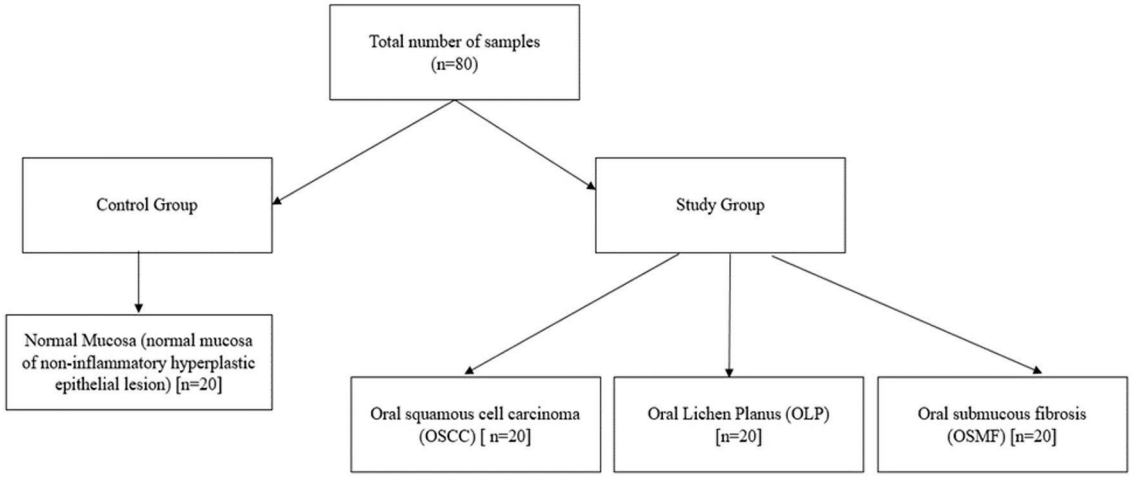


Figure 1. Patient flow diagram.

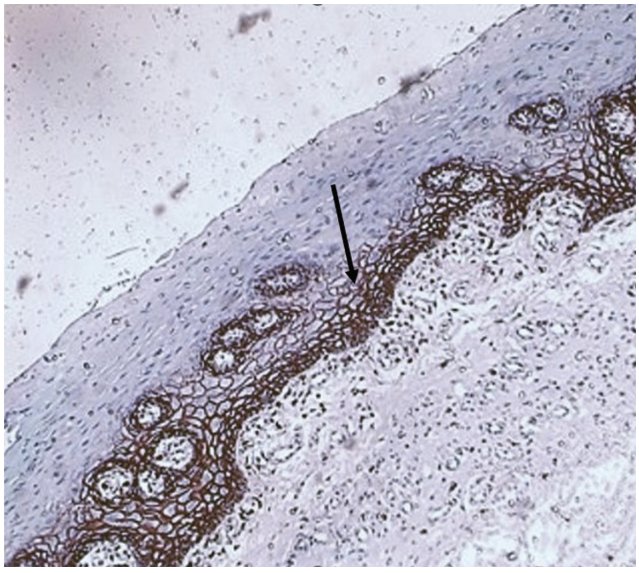


Figure 2. Using immunohistochemistry, the CD44 immunopositivity in normal oral mucosa (Control) x100 magnification.

the 80 cases showed immunopositivity for CD44. Brown staining in the cytoplasm of the epithelial cells was an indication of the immune response in the control tissue (Figure 2). The degree of expression was seen from the basal to the spinous cell regions. In OLP, positive CD44 immuno-expression was evident in the cytoplasm/nucleus of the basal and suprabasal layers of epithelium (Figure 3). There was also evidence of CD44 expression seen in the chronic inflammatory cells in the connective tissue stroma of lichen planus cases. In OSMF, CD44 expression was seen in the cytoplasm/nucleus of the basal and spinous layers of epithelium (Figure 4). In OSCC, CD44 immuno-expression was seen in the cytoplasm of the neoplastic cells surrounding the keratin pearls and in the lymphocytes (Figure 5). There was no inter-observer variability in interpreting the slides.

The descriptive statistics were given (Table 1). P value was <0.001 when compared to the groups. Comparison within the groups were done using the Independent Student's t-test. Significant p-value was found on comparison between control and OLP, control and OSMF,

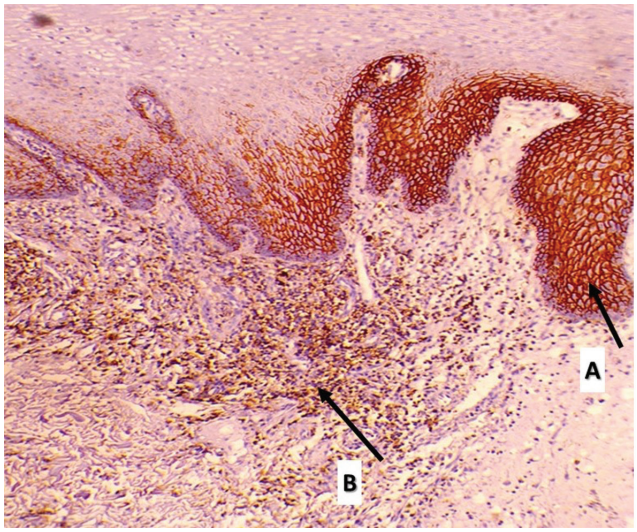


Figure 3. Using immunohistochemistry, the CD44 immunopositivity in oral lichen planus x100 magnification. The arrow marks in the figure denotes CD44 immuno-expression in the cytoplasm/nucleus of the basal and suprabasal layers of epithelium (A) and the chronic inflammatory cells in the connective tissue stroma of lichen planus (B).

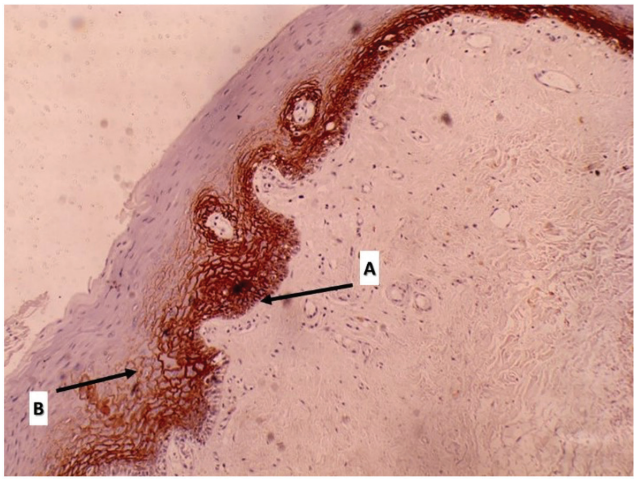


Figure 4. Using immunohistochemistry, the CD44 immunopositivity in oral submucous fibrosis x100 magnification. The arrow marks in the figure denotes CD44 expression in the cytoplasm/nucleus of the basal (A) and spinous layers (B) of epithelium.

control and OSCC, OLP and OSCC and among OSMF and OSCC. Nevertheless, the comparison between OLP and OSMF revealed no statistically significant (Figure 6).

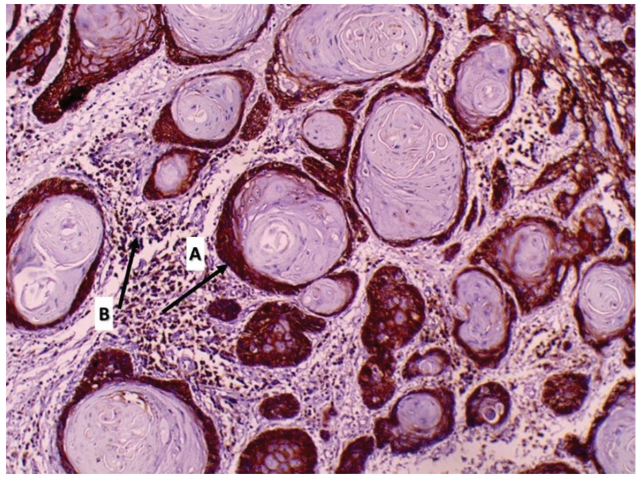
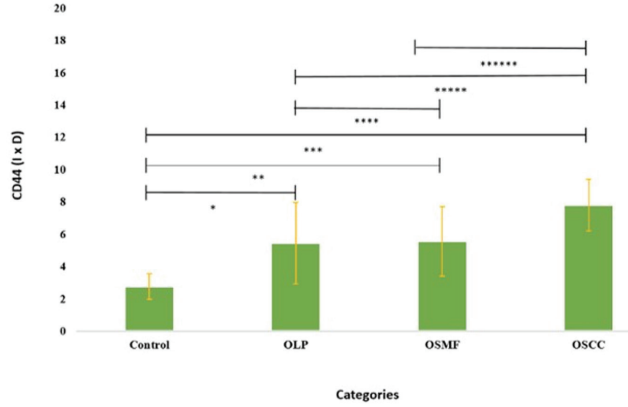


Figure 5. Using immunohistochemistry, CD44 shows strongly positive in a case of oral squamous cell carcinoma x100 magnification. The arrow marks in the figure refers the CD44 immuno-expression in the cytoplasm of the neoplastic cells surrounding the keratin pearls (A) and in the lymphocytes (B).



*	P = 0.000
**	P = 0.000
***	P = 0.000
****	P = 0.894
*****	P = 0.001
******	P = 0.001

Figure 6. CD44 expression in control, OLP, OSMF and OSCC.
OLP: Oral lichen planus, OSMF: Oral submucous fibrosis, OSCC: Oral submucous fibrosis

Discussion

Around 1805, European physicians labelled OPMDs as "pre-cancer", suggesting that these benign conditions would eventually develop into severe malignancies (9). To facilitate timely detection as well as follow-up in the event of ambiguous abnormalities and lower the likelihood of a malignant transformation, early identification of lesions is essential. The term "oral potentially malignant disorders" OPMDs appear to relate to OLP, OSMF. OSCC may develop in the absence of prompt detection and treatment of OPMDs (15). The ability of histological diagnostics to determine the kind of oral lesion and its susceptibility

for malignancy is currently acknowledged as a valuable tool (16). Despite attempts to improve OPMD care, diagnosis is made very late in majority of cases, making it exceedingly difficult to find a cure and having little effect from medications. To prevent OPMDs from turning into malignancies, it is essential to identify them as soon as possible, especially in high-risk groups (17).

The multistructural and multifunctional cell surface protein known as CD44 is responsible for mediating a number of biological processes, including angiogenesis, cell proliferation, differentiation, migration, presentation of cytokines, chemokines, and growth factors to the proper receptors, docking of proteases at the cell membrane, and signaling for cell survival. Many of these biological characteristics are linked to the pathologic actions of cancer cells, but they also have a big impact on how normal cells behave physiologically (18).

Taking into account all of these data, the current study was carried out to determine whether there had been any appreciable changes in the expression of CD44 among the normal mucosa, OLP, OSCC and submucous fibrosis.

According to our findings, the basal and intermediate layers of healthy oral mucosa as well as the epithelial cell membrane, all exhibited prominent CD44 protein expression (Figure 2). As it is well known, the CD44 protein is a member of the stem cell family and characterizes epithelial localization, the mediating of sticky qualities, and signals for epithelial cell migration in an (19,20). The normal mucosa exhibited mild-intensity staining of CD44, while abnormal cases displayed elevated expression.

In the current investigation, CD44 expression was lowest in the cells in the control group then OLP, followed by OSMF, and greatest in OSCC (Control<OLP<OSMF<OSCC).

This is not in line with the research of Zargaran et al. (21), whose findings indicated that oral squamous cell carcinoma expressed less CD44 than that of OLP. They suggested that it could indicate that the cells in OSCC group are more prone to the cleavage of its CD44 extracellular domain. Its cleavage disables Merlin's activity, eliminates cell cycle arrest, and results in unchecked cell growth (21). Abdal et al. (22) in their study also described that lichen planus had greater CD44 expression than OSCC in his research.

The findings of Dalley and Mannelli's study (23,24) has demonstrated that the expression of the CD44 marker rose-in OSCC lesions, which is consistent with the current study. This could be as a result of expression of CD44, an activation marker linked to proliferation and angiogenesis, being expressed in OSCC. Moreover, it has been demonstrated that CD44 was involved in blood vessel development and that inhibiting CD44 activity inhibits tumor and wound angiogenesis (25).

In the current study, OSMF expression was slightly higher than OLP but less than OSCC. This might be because, as a result of epithelial atrophy, since OSMF epithelial cells are highly differentiated, it might result in strong staining of CD44. These results raise the possibility that epithelial maturation might be different in OMSF and OLP or that their expression might be indicative of cancerous potential. This agrees with the conclusions reached by Dhumal et al. (26).

When patients with OSMF and OLP were compared, the CD44 expression did not show a statistically significant difference. However, a statistically significant difference was observed between patients with OLP and OSCC, a well as between OSMF and OSCC. This is in accordance with the research conducted by Abdal et al. (22), who studied two OPMDs and discovered no differences between OLP and oral leukoplakia (27).

According to the study conducted by Ghazi et al. (28), the tissue in OSCC group exhibited the highest levels of CD44 expression, while the ones in control group showed the lowest levels. This outcome is consistent with our research.

This suggests that CD44 may serve as a indicative marker for tracking the transition from healthy mucosa to premalignant and ultimately to malignant tissue, based on the varying levels of CD44 expression identified through immunohistochemistry.

Study Limitations

Due to time constraints, the study had certain limitations, such as a decreased sample size and the inability to assess the histological grades of each group. Given the retrospective nature of the study, patient histories were solely acquired through data files, which leads to bias in patient histories. Hence, more studies with a bigger sample size, varied tumor grading, and a prospective study are suggested to further validate CD44 as a prognostic marker.

Conclusion

Based on all of these observations, we conclude that the tissues of patients with OSCC has much greater levels of CD44 expression than those of OSMF, OLP and the control group, suggesting that malignant cells are more likely to express CD44 than those of OPMDs and normal mucosa. CD44 has an important role in cell-cell and cell-matrix interaction as well as cell migration and tumor progression. So, increased expression of CD44 would increase the risk of malignancy. Hence, CD44 can be employed as a indicative marker to assess the propensity for malignancy in OPMDs and similarly for OPMDs in normal mucosa. Nevertheless, more research with a bigger sample size and perhaps with various grades in each lesion is warranted to define the diagnostic importance of CD44 expression.

Ethics

Ethics Committee Approval: The study received approval from the Institutional Review Board (approval number: SRMU/M&HS/SRMDC/2022/PG/012 - SRM Dental College) on June 29, 2022.

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: S.R., N.G., A.J., T.V., R.K., Design: S.R., N.G., A.J., T.V., R.K., Data Collection or Processing: S.R., N.G., A.J., T.V., R.K., Analysis or Interpretation: S.R., N.G., A.J., T.V., R.K., Literature Search: S.R., N.G., A.J., T.V., R.K., Writing: S.R., N.G., A.J., T.V., R.K.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declare that they have no relevant financial.

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