Original Article

Immunohistochemical expression of putative cancer stem cell markers aldehyde dehydrogenase 1, SOX2, CD44 and OCT4 in different grades of oral epithelial dysplasia

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Abstract

Objectives: The hypothesized existence of cancer stem cells (CSC) and its markers aldehyde dehydrogenase 1 (ALDH1), CD44, SOX2 and OCT4 in oral dysplastic tissues provides the potential for a more reliable assessment of malignant transformation of oral epithelial dysplasia (OED). Thus, the present study is intended to evaluate the immunohistochemical expression of four different CSC markers ALDH1, CD44, SOX2 and OCT4 in different grades of OED and to investigate the co-expression of these putative stem cell markers in OED.

Subjects and Methods: A total of 35 samples of varying grades of OED which included 7 mild, 11 moderate and 17 severe dysplasia samples and 10 samples of normal oral mucosa without dysplasia were used. Four sections each from all 45 samples were stained with ALDH1, CD44, SOX2 and OCT4, respectively, by immunohistochemistry. The acquired data were analyzed and evaluated using the Chi-square test and unpaired *t*-test and the $P < 0.05$ was taken significant.

Results: ALDH1 and SOX2 expression percentages showed statistically significant differences among study groups ($P < 0.05$). Statistical comparison of percentage expression of CD44 and OCT4 between OED and normal was nonsignificant ($P > 0.05$). Co-expression of all four markers was found in 15 cases of OED with none of the normal cases showing co-expression.

Conclusion: The expression of CSC markers in OED and normal mucosa differs significantly with co-expression of all four markers located only in dysplastic tissues. Until now, no single protein marker has been able to unequivocally identify the CSCs. Thus, a panel of putative CSC markers will help in identifying the patients with high risk for malignant transformation in OED.

Keywords: Aldehyde dehydrogenase 1, cancer stem cells, CD44, OCT4, oral epithelial dysplasia, SOX2

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INTRODUCTION

Despite evolutions in the field of medicine, head-and-neck squamous cell carcinoma (HNSCC) remains to be one of the most common malignancies worldwide and the leading cause of mortality among males in India.[1] Oral squamous cell carcinoma (OSCC) has a fairly onerous prognosis, with an overall 5-year survival rate ranging from 40% to 58% due to late diagnosis, metastatic behavior and recurrence potential, thus encouraging further research on factors that might modify the disease outcome.[2]

Oral cancer affects 20/100,000 people in India, accounting for about 30% of all cancers.[3] Most cases of oral cancer are associated with deleterious habits (tobacco/areca nut) and are anteceded by asymptomatic clinical lesions referred to collectively as oral potentially malignant disorders (OPMD)[4] which include leukoplakia, erythroplakia, erosive lichen planus, reverse smoker's palate, oral submucous fibrosis (OSF), actinic keratosis and lupus erythematosus.[5,6] OPMD is a clinical diagnosis that may exhibit hyperplasia, hyperkeratosis, oral epithelial dysplasia (OED) or OSCC on histological examination. The presence of OED is the most accurate predictor of OPMDs with a higher risk of malignancy progression. OED is characterized by cytological and architectural variations that represent the lack of normal surface epithelium maturation and stratification patterns.[7,8] Various studies reported a malignant transformation rate of 0.13%–34% in OPMDs.[9]

The progression of OPMDs to OSCC is found to be influenced by several factors, and hence, there is a need for better understanding and prognostication of malignant transformation to curb the disease at an initial stage. Researches imply that the initiation, progression, metastasis and recurrence of HNSCC are related to the behavior of cancer stem cells (CSCs) which are defined as a subpopulation of cells that exhibit self-renewal capacity with the ability to produce a heterogeneous lineage of cells that comprise the tumor.[10] CSC moreover possesses the ability to differentiate and regenerate thus contributing resistance to chemotherapy and radiation treatments in tumors.[11] Thus, investigation of CSCs markers and their selective targeting is the focus of cancer research nowadays.

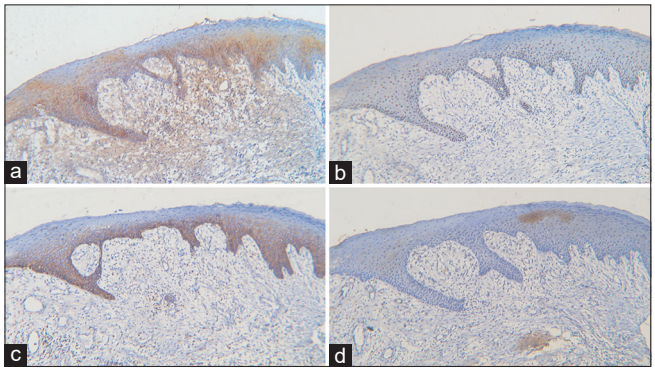

Figure 1: Immunoexpression of cancer stem cells markers in mild oral epithelial dysplasia: Photomicrographs showing positive immunohistochemical expression of aldehyde dehydrogenase 1 (a), SOX2 (b), CD44 (c) and OCT4 (d) in mild oral epithelial dysplasia

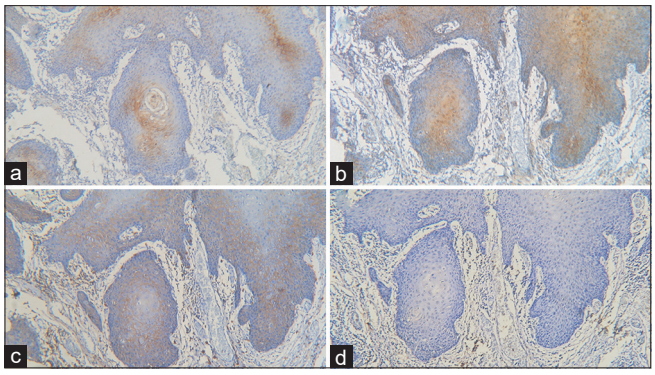

Figure 3: Immunoexpression of cancer stem cells markers in severe OED: Photomicrographs showing positive immunohistochemical expression of aldehyde dehydrogenase 1 (a), SOX2 (b), CD44 (c) and OCT4 (d) in severe oral epithelial dysplasia

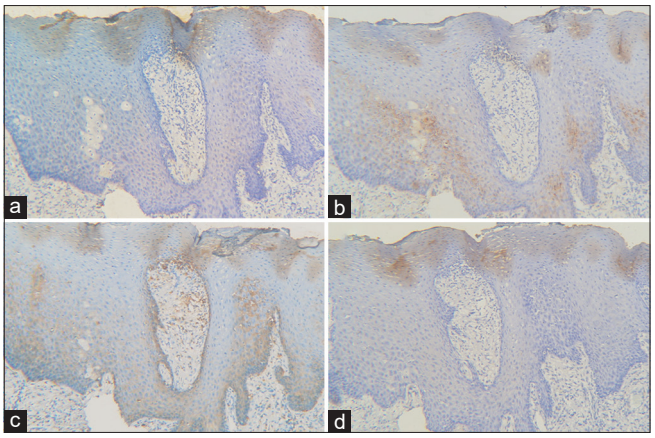

Figure 2: Immunoexpression of cancer stem cells markers in moderate OED: Photomicrographs showing positive immunohistochemical expression of aldehyde dehydrogenase 1 (a), SOX2 (b), CD44 (c) and OCT4 (d) in moderate oral epithelial dysplasia

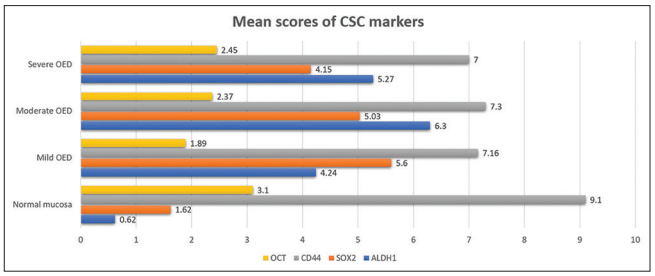

Figure 4: Graphical representation of mean immunoscore of cancer stem cells markers: Graph showing mean immunohistochemical expression score of aldehyde dehydrogenase 1, SOX2, CD44 and OCT4 in normal mucosa, mild oral epithelial dysplasia, moderate oral epithelial dysplasia and severe oral epithelial dysplasia

The hypothesized existence of CSC in oral dysplastic tissues also provides the potential for a more reliable assessment of malignant transformation of OPMDs.[12]

Many protein markers have been studied as well-known CSC markers in OSCC samples and cell lines, including SOX2, OCT4, NANOG, CD44, CD133, CD24 and ALDH1.[13-17] Until now, no single protein marker could unambiguously identify the CSCs.[10,11] Hence a combination of markers is necessary to find the CSC subpopulation within tumor cells. Thus, this study intended to investigate four different molecular markers in patients with OED based on CSC theory.

Aldehyde dehydrogenase (ALDH) is a class of intracellular cytosolic iso-enzymes found mainly in the liver. Their recognized functions include conversion of retinol to retinoic acid during early stem cell differentiation and catalyzing the oxidation of toxic intracellular aldehyde metabolites into carboxylic acid, similar to those created by alcohol metabolism and chemotherapeutics.[18] ALDH1, a member of the ALDH enzyme family, has been identified as the primary ALDH isozyme associated with stem cell populations. ALDH1 is hypothesized to have a role in the malignant transition of oral leukoplakia to OSCC since ALDH1+ leukoplakia is more than three times more likely to develop OSCC.[19] CD44 is the major receptor for hyaluronan and constitutes a single chain transmembrane glycoprotein that is widely expressed in physiological and pathological conditions.[20] CD44 maintains tyrosine kinase activity and serves as an adhesion molecule by interacting with hyaluronan and cytoskeletal components.[21,22] Aberrant expression of variable CD44 isoforms in malignancy can lead to tumor extension and metastasis.[23] The tissue CD44 expression patterns in HNSCC and their relationship to prognosis have remained unclear in the literature, with many authors showing increased expression correlated with poor prognosis, while some suggesting improved prognosis.[22,24]

The stemness marker SOX2, one of the members of the SRY-related high mobility group box transcription factors family, stimulates reprogramming of adult stem cells into induced pluripotent stem cells and maintains stemness in cancer by complexing with other markers, having a key role in tumorigenesis and progression.[25] Studies showed an increase in mean expression of SOX2 from OED to OSCC thereby identifying its role in proliferative potential and transformation of OED into OSCC.[26] OCT4 is a component of the family of Pit-Oct-Unc domain transcription factors known to bind in partnership with SOX2 and acts as a key regulator essential for the self-renewal and pluripotent capacity of CSCs.[27-29] OCT4 has also been noted to be increased in OED.[30]

The present study intended to evaluate the immunoexpression of four different CSC markers CD44, ALDH1, OCT4 and SOX2 in varying grades of OED thus eliciting their role in oral carcinogenesis cascade, which could potentially impact and guide treatment. This study was also meant to investigate the co-expression of these putative CSC markers in OED.

SUBJECTS AND METHODS

Sample collection

A total of 35 oral biopsy samples with a histopathologic diagnosis of epithelial dysplasia and 10 samples of normal oral mucosa reported in the year 2018 were chosen from the archives of the Department of Oral and Maxillofacial Pathology. For normal samples, noninflamed tissue specimens obtained during dental surgical treatments were used. Tissue specimens obtained from patients with adverse habits and inflamed or dysplastic tissues were not included for normal samples. The sample size was calculated with a 95% confidence interval and 10%–20% of relative precision. The study material consisted of Formalin-Fixed, Paraffin-embedded (FFPE) samples from cases selected on the basis that eligible specimens should include an adequate area of dysplastic tissue and normal adjacent mucosa with at least one peripheral margin of the biopsy specimen. Patients' clinical records were reviewed to gather information on demographic details. According to institutional regulations, the study was approved by the institutional human ethics committee.

Histopathologic analysis and grading

Two independent pathologists analyzed representative hematoxylin and eosin sections of incisional biopsy specimens from each patient and confirmed the diagnosis. The cases were classified as severe, moderate and mild dysplasia based on the grading criteria in the 2017 WHO classification of head-and-neck tumors.[31] Consequently, 17 cases of severe dysplasia, 11 cases of moderate dysplasia and 7 cases of mild dysplasia were included in the study.

Immunohistochemistry procedure

FFPE sections were cut with 4-μm thickness and mounted on positively charged slides (FLEX immunohistochemistry [IHC] Slides K802021). IHC kit (EnVision ™ FLEX Mini Kit, High pH K8023) from DAKO, Agilent Technologies, was utilized for the study. Deparaffinization in xylene was done, followed by rehydration in graded alcohols and distilled water. Following

antigen retrieval with Tris/ethylenediaminetetraacetic acid buffer, pH9 (×50) using pressure boiler at 95°C for 40 min (min), the sections were washed with tris buffer for 5 min. Endogenous peroxidase activity was blocked using peroxidase-blocking reagent for 30 min. Slides were washed in 2 changes of tris buffer for 5 min each and incubated with the following Ready to Use primary antibodies from Master Diagnostica (Vitro, Spain): Goat anti-human ALDH1A1 Polyclonal (MAD-000611QD-R-3), Rabbit anti-human CD44 Monoclonal (MAD-000537QD-R-3), Rabbit anti-human SOX2 Monoclonal (MAD-000521QD-R-3) and Mouse Anti OCT3/4 Monoclonal Antibody (MAD-000239QD-R-3) for 30 min at 37°C in a humidifying chamber. Incubation with a secondary antibody conjugated with horseradish peroxidase was added to the slides at room temperature and kept for 30 min. The sections were washed in tris buffer twice for 4 min and visualization was performed using 3,3'-diaminobenzidine tetrachloride solution for 10 min. The sections were then counterstained with Mayer's hematoxylin. Immunohistochemical staining was carried out similarly for the negative control, but with the primary antibody being replaced with tris buffer. The positive controls for CD44, ALDH1, OCT4 and SOX2 consisted of tissue sections of tonsil, stomach, seminoma and normal skin, respectively.

Immunohistochemistry scoring

A Labomed LX500 light microscope (Labomed Inc., USA) was used to perform a semiquantitative analysis of immunoexpression of antigens in cells. Dark brown staining in the cytoplasm of epithelial cells was considered positive for ALDH1 expression and nuclear staining was considered positive for SOX2 and OCT4 expression. Cell membrane immunoreactivity of epithelial cells was considered positive for CD44 expression. All of the slides were evaluated by 2 observers independently.

The antibody expression was assessed in the epithelial cells under ×40 magnification using the following criteria. In five high-power fields, the proportion of positive cells was recorded and calculated as positive number of cells expressed in a field/total number of cells ×100. The percentage of positive cells was categorized as: 0% = score 0; <25% = score 1; 25%–49% = score 2; 50%–74% = score 3 and 75%–100% = score 4. Intensity scores for antibody expression were as follows: 0, no staining/negative; 1, weak staining; 2, intermediate/moderate staining and 3, strong staining. The final immunoscore was obtained by multiplying the percentage score and intensity score. The immunograding for the antibodies was given as (i) lower expression, ≤1 point; (ii) high expression, ≥2 points. In cases of any disagreement, the slides were re-examined to obtain a consensus.

Statistical analysis

The data were expressed in number, percentage, mean, median and standard deviation. For the study analysis, the statistical package for the social sciences version 20.0 (SPSS Inc, IBM Chicago, USA) was utilized. At a 95% confidence interval, a $P < 0.05$ was considered statistically significant using the unpaired *t*-test and Chi-square test.

RESULTS

Demographic findings

The present study consisted of histologically proven 35 cases of varying grades of OED and 10 cases of normal mucosa. The demographic data of cases in the study are given in Table 1. The study group included 88.57% males (31/35) and 11.43% females (4/35) in the age group 42–80 years with a mean age of 62.37 years. The lesions were diagnosed clinically as leukoplakia (42.86%), erythroplakia (40%) and OSF (17.14%). All the OSF cases were confirmed as varying grades of OED histopathologically. Most of the OED cases selected had an occurrence in the buccal mucosa (77.14%) followed by lip (17.14%) and tongue (5.72%). The 35 cases of OED comprised 20% (7/35) mild epithelial dysplasia, 31.43% (11/35) moderate epithelial dysplasia and 48.57% (17/35) severe epithelial dysplasia.

Immunohistochemistry findings

Percentage expression of CD44, aldehyde dehydrogenase 1, OCT4 and SOX2 in oral epithelial dysplasia and normal mucosa

The percentage expression of CD44, ALDH1, OCT4 and SOX2 in study groups has been tabulated in Table 2. ALDH1 and SOX2 expression percentages showed statistically

Table 1: Demographic data of cases included in the oral epithelial dysplasia group

Parameters	Number of patients (*n*=35)
Age (years)	
40-50	4
51-60	11
61-70	13
71-80	7
Gender	
Male	31
Female	4
Clinical appearance	
Leukoplakia	15
Erythroplakia	14
OSMF	6
Site	
Buccal mucosa	27
Labial mucosa	6
Tongue	2
Dysplasia	
Mild	7
Moderate	11
Severe	17

OSMF: Oral submucous fibrosis

significant differences among study groups ($P < 0.05$). Statistical comparison of percentage expression of CD44 and OCT4 between OED and normal was nonsignificant ($P > 0.05$). The expression of CD44 was increased in normal mucosa than that of OED though the result was statistically not significant. All 10 normal cases showed positive CD44 expression with a median value of 9.9.

Comparison of mean final score of expression of CD44, aldehyde dehydrogenase 1, OCT4 and SOX2 in different grades of oral epithelial dysplasia

The mean expression of ALDH1 was more in moderate dysplasia (6.30 ± 4.36), followed by severe dysplasia (5.27 ± 3.05). The mean score of SOX2 was more in mild dysplasia (5.6 ± 4.6) followed by moderate dysplasia (5.03 ± 3.5). Furthermore, statistically similar levels of CD44 expression occurred in all stages of oral dysplasia regardless of mild, moderate or severe dysplasia. The mean expression value of OCT4 was more in severe dysplasia (2.45 ± 2.85) followed by moderate dysplasia (2.37 ± 2.67).

The median values of CD44, ALDH1, OCT4 and SOX2 immunoscores in OED and normal mucosa and their mean expression in mild, moderate and severe dysplasia are given in Table 3.

Pattern of expression of aldehyde dehydrogenase 11, CD44, SOX2 and OCT4 in varying grades of oral epithelial dysplasia

Immunoxpression of these markers was limited to basal as well as few parabasal layers in positive normal cases, but dysplastic epithelium showed expression up to superficial layers. In the case of mild dysplasia, expression of ALDH1 was seen only in spinous and superficial layers whereas, in moderate and severe dysplasia cases, the expression was also noted in the basal layer of dysplastic epithelium. The expression of SOX2 was seen throughout the epithelium in mild dysplasia, while moderate and severe cases showed expression only in basal and spinous layers of the dysplastic epithelium. Immunoexpression of cancer stem cells markers in mild, moderate and severe grades of oral epithelial dysplasia are shown in Figures 1 to 3 respectively. Meanwhile, CD44 expression was noted in all layers of the dysplastic epithelium in all stages of OED. Expression of OCT4 was seen only in spinous and superficial layers in case of mild dysplasia, whereas in moderate and severe dysplasia cases, the expression was noted in all layers of the dysplastic epithelium.

Co-expression of markers in oral epithelial dysplasia and normal mucosa

The co-expression of CD44, ALDH1, OCT4 and SOX2 proteins was assessed in the same sample by assessing the positivity of immunoexpression within the identical areas of the tissue specimen. Co-expression of all four markers was located in 15 cases of OED in that 4 mild, 4 moderate and 7 severe OED cases were present. Graphical representation of mean immunoscore of cancer stem cells marker expression is given in Figure 4. Furthermore, co-expression of ALDH1 and CD44 was found in a total of 22 OED cases and co-expression of SOX2 and OCT4 was found in 19 OED cases. None of the normal cases showed co-expression of these markers.

DISCUSSION

The incidence of oral cancer has been rising in many countries. Many OSCC cases are preceded by clinically

Table 2: Percentage expression of aldehyde dehydrogenase 1, SOX2, CD44 and OCT4 in oral epithelial dysplasia and normal mucosa

Study group	Sample size (*n*)	Percentage expression of CSC marker							
		ALDH1		SOX2		CD44		OCT4	
		Positive, *n* (%)	Negative, *n* (%)	Positive, *n* (%)	Negative, *n* (%)	Positive, *n* (%)	Negative, *n* (%)	Positive, *n* (%)	Negative, *n* (%)
Normal	10	1 (10)	9 (90)	2 (20)	8 (80)	10 (100)	0	4 (40)	6 (60)
Dysplasia	35	25 (71.43)	10 (28.57)	21 (60)	14 (40)	29 (82.86)	6 (17.14)	14 (40)	21 (60)
Mild dysplasia	7	3 (42.86)	4 (57.14)	5 (71.43)	2 (28.57)	5 (71.43)	2 (28.57)	2 (28.57)	5 (71.43)
Moderate dysplasia	11	9 (81.82)	2 (18.18)	7 (63.64)	4 (36.36)	9 (81.82)	2 (18.18)	4 (36.36)	7 (63.64)
Severe dysplasia	17	13 (76.47)	4 (23.53)	9 (52.94)	8 (47.06)	15 (88.24)	2 (11.76)	8 (47.06)	9 (52.94)

CSC: Cancer stem cells, ALDH1: Aldehyde dehydrogenase 1

Table 3: Immunoscores of aldehyde dehydrogenase 1, SOX2, CD44 and OCT4 in mild, moderate and severe oral epithelial dysplasia

Marker	OED		Normal		*P*	Mean±SD		
	Mean	Median (IQR)	Mean	Median (IQR)		Mild dysplasia (*n*=7)	Moderate dysplasia (*n*=11)	Severe dysplasia (*n*=17)
ALDH1	5.39	6 (6.48)	0.62	0.16 (0.66)	<0.001*	4.24±5.26	6.30±4.36	5.27±3.05
SOX2	4.71	3.6 (7.62)	1.62	1.24 (2.46)	0.012*	5.6±4.6	5.03±3.5	4.15±4.08
CD44	7.16	8.32 (7.6)	9.1	9.9 (5.1)	0.14	7.16±5.3	7.3±4.3	7±3.8
OCT4	2.31	1.4 (2.61)	3.1	1.2 (5.39)	0.722	1.89±2.27	2.37±2.67	2.45±2.85

*$P \leq 0.05$ is considered to be statistically significant. IQR: Interquartile range, SD: Standard deviation, ALDH1: Aldehyde dehydrogenase 1, OED: Oral epithelial dysplasia

evident OPMDs. A patch-field carcinoma progression model of oral cancer proposed by Braakhuis *et al*. hypothesized that oral cancer development starts with a "patch stem cell" and develops into an expanding subpopulation of stem cells that escape growth control and eventually result in malignant transformation.[32] CD44, ALDH1, OCT4, and SOX2 are well-studied CSC markers that have been implicated in several solid tumors including oral cancer.[19,24,27] To the best of our understanding, for the first time, our investigation determined to explore the pattern of expression of four different CSC markers in varying grades of OED in English literature. Thus, the panel of antigens evaluated has a range of cellular functionality, replicating the breadth of known CSC markers found in current literature.

ALDH1 has been shown to be elevated in a subset of HNSCC-derived CSCs as a CSC marker.[18] Visus *et al*. and Liu *et al*. reported that 32.5% and 38.3% of patients with OED showed ALDH1 expression, respectively.[19,33] In this present study, 71.43% of cases showed positive ALDH1 expression with higher mean scores in moderate and severe dysplasia. Liu *et al*. also reported that ALDH1 was closely linked to a worse malignant prognosis in individuals with oral leukoplakia.[19] Such a finding could suggest the role of ALDH in the stepwise alteration of OED to carcinomas.

CD44 is responsible for the adhesive characteristics of epithelial cells as well as signaling their upward migration. When compared to normal mucosa, CD44 expression was shown to be lower in three grades of epithelial dysplasia in the current study similar to the findings reported by Mack and Gires.[34] This might be due to early cellular alterations from normal cell-cell and cell-matrix interactions to the strange, pathophysiologic heterotypic cell surface adhesion feature, which could be a factor in cell invasion and early development of oral cancer.[34]

SOX2 and OCT4 together are key regulators for stem cell self-renewal and maintenance in undifferentiated tissue. In a study by Qiao *et al*., the percentage positivity of SOX2 was 90% and OCT4 was 70% in OPMDs.[29] The current study also reported 60% SOX2 and 40% OCT4 positivity. The expression of SOX2 in OED even though less but in comparison with normal mucosa showed significant difference, thereby identifying the proliferative potential and transformation of OED into OSCC, but OCT4 expression showed similar values in both normal mucosa and OED with no statistical significance.

The co-expression of these markers was defined as its positive expression on the same location within a tumor mass.[26] Co-expression of all four markers was found in 15 cases of OED in the present study with none of the normal samples showing co-expression. Similar to our study, Qiao *et al*. reported co-expression of OCT4 and SOX2 in 12/20 cases of OPMDs and also reported that individual expression of OCT4 and SOX2 was seen in the basal layer of normal mucosa, however, they did not demonstrate co-expression in any of the normal samples. The transcriptional factors, SOX2 and OCT4, are co-expressed in embryonic stem cells, but the double-positive co-expression profile of these markers cannot be demonstrated in normal mucosa.[29]

Interestingly, to the best of our knowledge, this is the first report in English literature to show the co-expression of CD44, ALDH1, OCT4 and SOX2 in OED. Bourguignon *et al*. reported that the expressions of OCT4 and SOX2 were related to CD44 and demonstrated that a subpopulation of CSCs overexpressing CD44 v3 and ALDH1 expressed SOX2, Nanog and OCT4 as well as unveiled the characteristic CSC traits of self-renewal/clonal formation and the ability to produce diverse cell populations.[35] Thus, co-expression of all these four markers will be helpful in finding the CSC subpopulation within the dysplastic epithelium.

Oral dysplastic lesions with a high risk of turning into oral cancer remain a therapeutic issue that, if overcome, would allow patients to benefit from early therapeutic intervention. There are presently no biomarkers that can be utilized to predict high-risk oral dysplastic lesions in the clinical setting. The link between putative CSC markers and OPMDs and oral epithelial tumorigenicity has already been shown, proposing that delineating the pattern of putative stem cell antigens might have significant prognostic and diagnostic relevance. So far, no single protein marker could unequivocally identify the CSCs. Thus, a panel of putative CSC markers will help the clinicians in identifying the patients with high risk for malignant transformation in OED. We acknowledge that the limited sample size of our study is a possible constraining factor and that additional studies with a larger patient cohort and follow-up analysis are needed to substantiate our findings.

CONCLUSION

There is a significant difference in expression of CSC markers in OED and normal mucosa with co-expression of all four markers located only in dysplastic tissues. This paves the way for the creation of a panel of potential CSC markers for the early detection of OED cases with a high risk of malignancy. Further studies with large sample size and follow-up analysis will authenticate these findings.

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Conflicts of interest

There are no conflicts of interest.

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