Original Article

Expression of CK 19 as a biomarker in early detection of oral squamous cell carcinoma

Parvathy Rajeswari[1], Mahija Janardhanan[1], Rakesh Suresh[1], Vindhya Savithri[1], Thara Aravind[1], Greeshma C Raveendran[2]

[1]Department of Oral Pathology and Microbiology, Amrita School of Dentistry, AIMS, Amrita Vishwa Vidyapeetham, [2]Department of Biostatistics, Amrita School of Medicine, AIMS, Amrita Vishwa Vidyapeetham, Kochi, Kerala, India

Abstract

Background: Increased mortality in oral squamous cell carcinoma (OSCC) had been attributed to delay in diagnosis. Lack of a specific marker to assess the malignant potential of premalignant lesions is thought to be one of the reasons for late detection. Expression of Cytokeratin 19, which is widely used as an odontogenic epithelial marker had been reported in OSCC. Downregulation of CK 19 expression plays an important role in terminal differentiation of superficial squamous cell and increased expression in various epithelial malignancies has been suggested to be an indicator of malignant change.

Aims and Objectives: To assess the role of CK19 as a potential marker in predicting malignant transformation in oral precancerous lesions and as a prognostic marker in OSCC.

Materials and Methods: Study population consisted of ten samples each of normal oral mucosa, epithelial hyperplasia, varying grades of both oral epithelial dysplasias and OSCC. The tissue sections were subjected to immunohistochemical staining for the marker cytokeratin 19.

Results: An increased expression of CK19 was noted in oral epithelial hyperplasia, severe dysplasia and in superficial epithelium at the invading front in OSCC. In mild and moderate dysplasias, CK19 expression was lower than the normal mucosa. In oral squamous cell carcinoma, the expression of CK19 was restricted to either a few islands or a few cells within the islands, resulting in a lesser expression than the normal epithelium. The malignant epithelial islands in the superficial connective tissue stroma were showing greater expression than the deeper islands. The epithelial cells associated with formation of keratin pearls were found to be showing more expression than those with infrequent keratin pearls.

Conclusion: The study suggests that malignant transformation of epithelium can be predicted based on the increased expression of CK19. But it should be done with caution as a similar increased expression may also be noticed in presence of inflammation..

Keywords: CK 19.Oral epithelial dysplasia, oral squamous cell carcinoma

Address for correspondence: Dr. Mahija Janardhanan, Department of Oral Pathology, Amrita School of Dentistry, AIMS, Amrita Vishwa Vidyapeetham, Amrita University, Kochi - 682 041, Kerala, India.
E-mail: mahijaj@yahoo.co.in

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity.[1] This can either develop as *de nevo* or can be preceded by a state of premalignancy known as "epithelial dysplasia," which is characterized by changes in architectural and cellular levels. The role of biomarkers in detecting epithelial dysplasias has been studied by various authors, including the expression of cytokeratins. CK19, an odontogenic epithelial marker, has also been reported to exhibit increased expression in various cancers, including OSCC and also in oral epithelial dysplasias. The studies carried out to assess the expression of CK19 in oral epithelial dysplasias and OSCC are limited in number and solid evidence regarding the authenticity and reliability of this marker is insufficient. Hence, it was planned to carry out a study to assess the expression of CK19 in various grades of oral epithelial dysplasias and OSCC and to evaluate their role as a potential marker in predicting malignant transformation in precancerous lesions.

MATERIALS AND METHODS

The study was conducted with approval from the Ethical committee, Amrita Institute of Medical Sciences and Research Centre and the study population consisted of patients diagnosed with hyperplastic epithelium, epithelial dysplasias, and squamous cell carcinomas. The paraffin-embedded blocks of hyperplastic tissue (n = 10), 10 cases of all three grades of oral epithelial dysplasia, OSCC and tissues obtained from normal oral mucosa were retrieved from the Archives of Department of Oral Pathology and Microbiology. The samples were categorized as Group I-IV. Samples included under oral epithelial dysplasia were graded according to the WHO 2005 classification system[2] and were found to satisfy all the criteria. Group IV comprising of OSCC were graded as well differentiated, moderately differentiated and poorly differentiated OSCC based on Broder's criteria.[3] Immunohistochemical staining using cytoskeletal marker CK19 was performed as per the standard protocol for indirect technique. Following clearing and dehydration, the tissue sections of 5-μ thickness were transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 15 min. After washing in PBS, endogenous peroxidase blocking was done by dipping sections in freshly prepared 3% H_2O_2 for 10 min. After blotting the excess peroxide, the slides were treated with a protein block reagent. Sections

Table 1: Scoring criteria - proportion score

Proportion score	Scoring criteria
0	No cells are +
1	≤1% cells are +
2	1%–10% of cells +
3	11%–33% of cells +
4	34%–66%
5	67%–100%

Table 2: Scoring criteria - intensity score

Intensity score	Scoring criteria
0	None
1	Weak
2	Intermediate
3	Strong

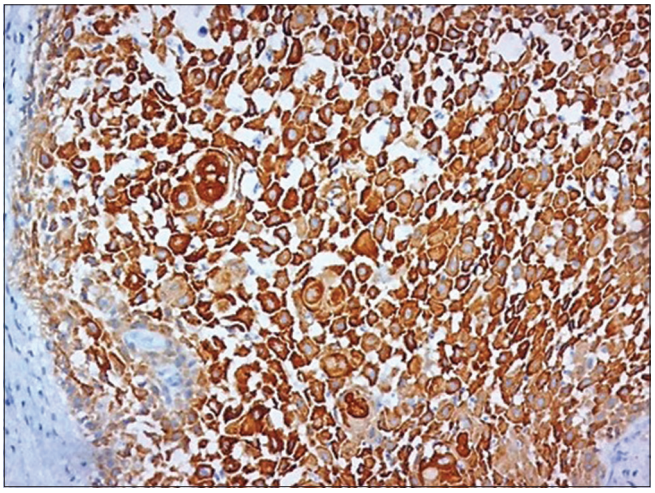

Figure 1: CK19 positive cells showing different hues of brown color in cytoplasm, IHC, ×10

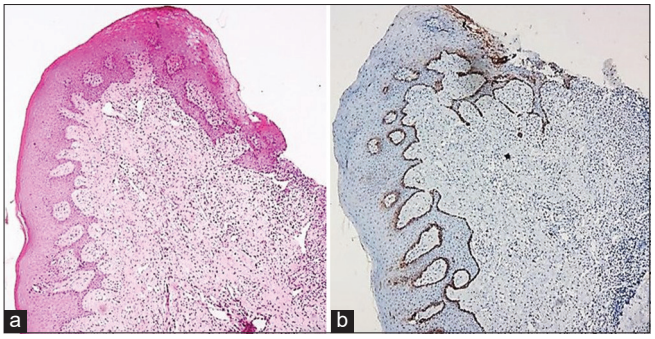

Figure 2: (a) Normal keratinized oral mucosa. H and E, ×4. (b) CK19 expression restricted to the basal layer. IHC, ×4

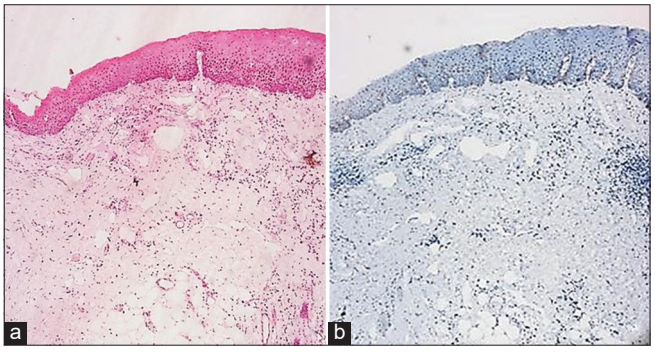

Figure 3: (a) Normal nonkeratinized oral mucosa. H and E, ×4. (b) CK19 expression restricted to the basal layer, IHC, ×4

were then incubated with primary antibody CK19 at room temperature for 1 h. The sections were taken out were washed in PBS (3 changes) for 5 min each to remove the excess antibody. A drop of enhancer from the secondary antibody kit (Pathinsitu Pvt. Ltd.) was added, and the slides were incubated for 30 min followed by the addition of a drop of Streptavidin from the secondary antibody kit on the sections and incubated for 30 min. The sections were washed in 3 changes of PBS for 5 min each, and a drop of freshly prepared DAB (3'diaminobenzidine tetrahydrochloride a substrate chromogen) was added on both sections. Slides were washed in PBS to remove excess DAB and then counterstained with hematoxylin. The tissue sections were mounted with DPX.

The sections were initially scanned at low power. A prominent brown cytoplasmic staining was considered positive for samples selected. The positively stained cells were scored using the Allred score[4] in 3 microscopic fields at ×40, that comprises proportion score and intensity scores as given in Tables 1 and 2.

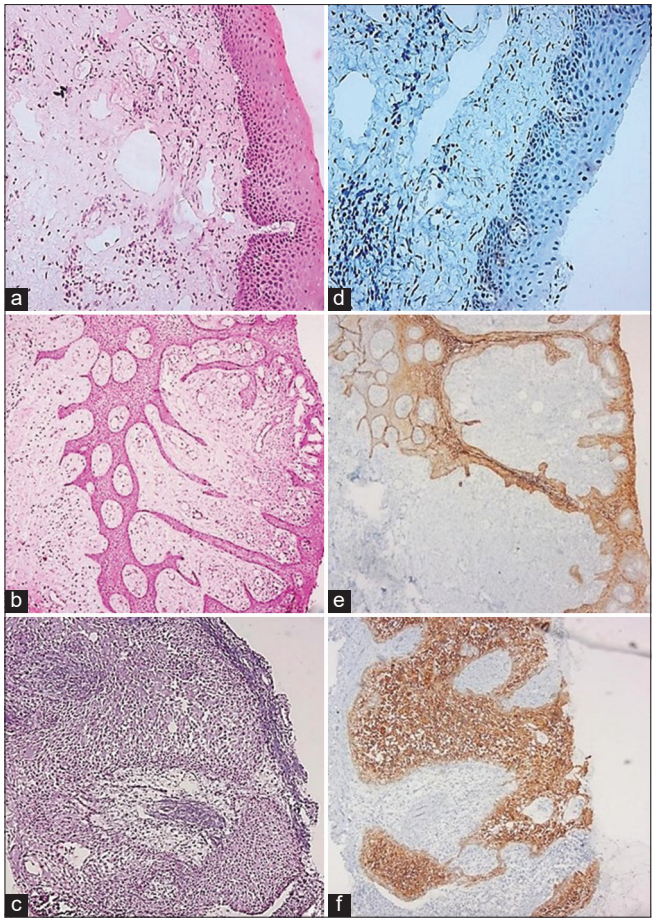

Figure 4: Comparison of CK19 expression in oral epithelial hyperplasia with varying degrees of inflammation (a-c). (a) Mild inflammation, (b) Moderate, (c) Dense inflammation. H and E, ×10. (d-f) Progressive increase in expression of CK19 with increase in grades of inflammation, IHC, ×10

RESULTS

The cytokeratin 19 expression was compared between four groups comprising control, oral epithelial hyperplasia, oral epithelial dysplasia and OSCC, which were grouped as Group I, II, III and IV, respectively. The CK19 expression was considered to be positive only in cells which showed different hues of brown color in the cytoplasm, denoting varying intensities of CK19 expression [Figure 1]. Apart from the positive control, the positive expression in salivary gland tissue was used as an internal control. In the control group of the normal oral mucosa, keratinized oral epithelium showed an increased expression of CK19 when compared to the nonkeratinized epithelium. CK19 expression was restricted to the cytoplasm of cells in the basal layer in both keratinized [Figure 2] and nonkeratinized epithelia [Figure 3]. Increased CK19 expression was noticed in focal epithelial hyperplasias associated with increasing grades of inflammation in both types of epithelia. The mean Allred score in group I was 5.40. In oral epithelial hyperplasia, a full-thickness positivity was noticed in nonkeratinized epithelial hyperplasia, whereas a positive expression was noticed involving the cells of basal, parabasal, spinous and some superficial cells in the rest of the samples. A similar increased expression of CK19 was noticed with increasing grades of inflammation [Figure 4]. The mean Allred score in this group was calculated as 10.50. CK19 expression was evaluated in all 30 cases of oral epithelial dysplasias. In mild dysplasia, out of 10 samples, 7 samples showed positive expression of CK19 and was seen as intermittent moderate-to-strong brown color in the cells of basal and some cells in parabasal layers [Figure 5]. The mean proportion score of mild dysplasia was 3.1 and the mean intensity score was 1.7. The intensity of CK19 expression was detected to be more pronounced in areas of dense inflammation associated with epithelial hyperplasia. In moderate dysplasia, 6 cases out of 10 showed patchy discontinuous faint cytoplasmic brown-colored staining involving the cells of basal and parabasal layers of the epithelium [Figure 6]. The expression of CK19 was restricted to basal and parabasal layers as with mild dysplasia, but the

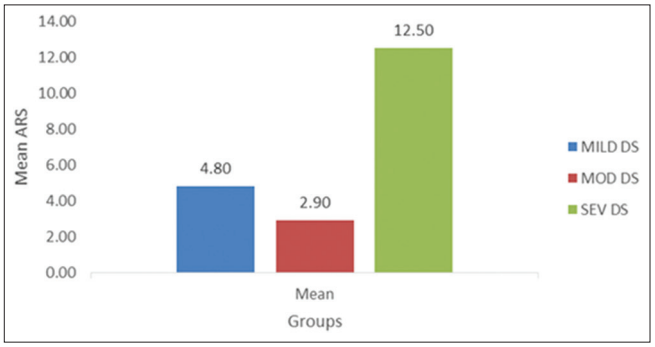

Diagram 1: Comparison among grades of oral epithelial dysplasia

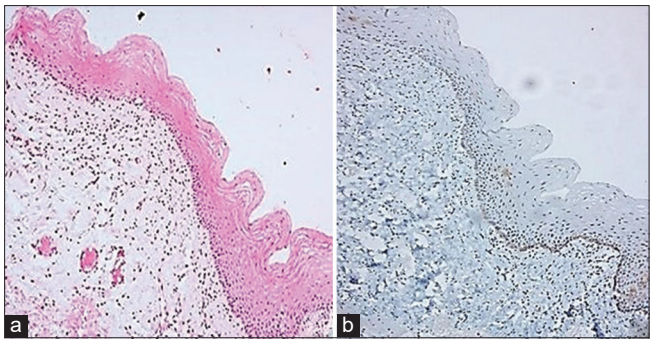

Figure 5: Mild epithelial dysplasia. (a) H and E, ×10. (b) CK19 expression restricted to basal and some cells in parabasal layers of epithelium, IHC, ×10

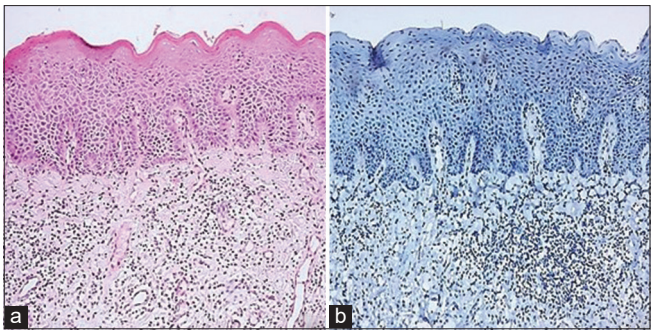

Figure 6: Moderate epithelial dysplasia. (a) H and E, ×10. (b) CK19 expression restricted to basal and some cells in parabasal layers of epithelium, IHC, ×10

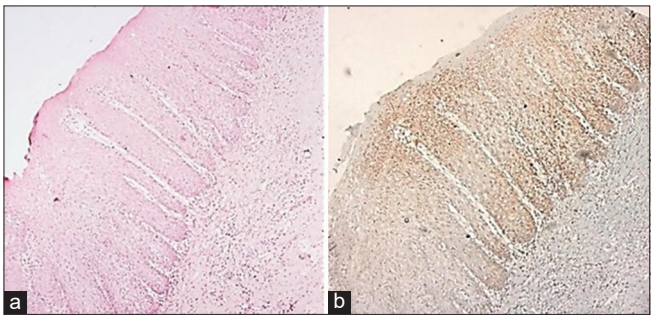

Figure 7: Severe epithelial dysplasia. (a) H and E, ×20. (b) CK19 expression involving cells of basal, parabasal, some spinous and superficial cells, IHC, ×20

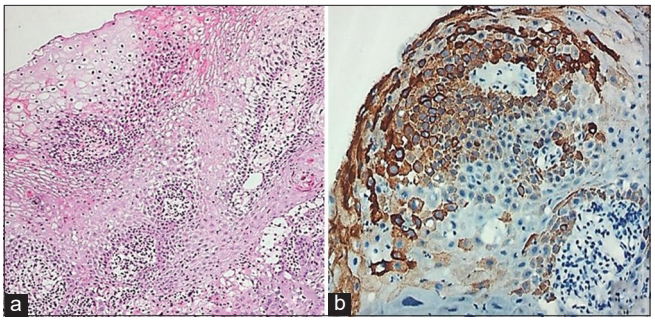

Figure 8: Superficial epithelium at the invasive front in well differentitated oral squamous cell carcinoma. (a) H and E, ×20. CK19 expressions in the same area (b) IHC, ×20

number of positive cells was less in moderate dysplasia. The mean proportion score of moderate dysplasia was 1.5 and the mean intensity was 1.4. In severe dysplasia, 9 samples showed an intense uniform strong cytoplasmic positivity involving the cells of basal, parabasal and spinous and some superficial cells also [Figure 7]. One sample showed a full-thickness positivity. The mean proportion score of severe dysplasia was 7. 40 and the mean intensity score was 5.10. The mean Allred score was calculated to be 6.73 in Group III. Statistical analysis using the Kruskal–Wallis test showed that the difference in mean value was found to be statistically significant, with the $P = 0.007$ [Table 3]. A bar diagram showing a comparison of mean Allred score among various grades of oral epithelial dysplasia is shown in Diagram 1.

In OSCC, out of 10 samples of well-differentiated OSCC, 8 samples showed positive expression of CK19 in the superficial epithelium. An obvious difference in expression was noticed in the expression of CK19 in the areas of invasion and the rest of the areas. In areas, other than the invasive front, the expression of CK19 was seen to be cytoplasmic and was distributed as focal interspersed positivity involving the cells of basal and occasionally parabasal layers. The superficial epithelium at the areas of invasion showed a stronger cytoplasmic positivity involving the basal, parabasal and superficial layers [Figure 8].

Table 3: Comparison of mean Allred score scoring index in various grades of oral epithelial dysplasia using Kruskal–Wally analysis

III sub groups	*n*	Mean	SD	*P*
Mild dysplasia	10	4.80	3.490	0.007
Moderate dysplasia	10	2.90	2.846	
Severe dysplasia	10	12.50	7.692	

SD: Standard deviation

Table 4: Comparison of mean Allred score scoring index in various grades of oral squamous cell carcinoma using Kruskal–Wally analysis

IV sub groups	*n*	Mean	SD	*P*
Well OSCC	10	1.80	1.989	0.244
Moderate OSCC	10	2.20	2.251	
Poor OSCC	10	3.50	2.877	

SD: Standard deviation, OSCC: Oral squamous cell carcinoma

The invading malignant epithelial islands showed varied expression of CK19 in different grades of OSCC. A comparison of CK19 expression between well, moderately differentiated and poorly differentiated OSCC is given in Figure 9. In well-differentiated OSCC, most of the islands showed negative expression of CK19. A few islands in superficial connective tissue stroma showed positive CK19 expression. Since a difference was noticed in CK19 expression between the well-differentiated islands showing keratin pearls

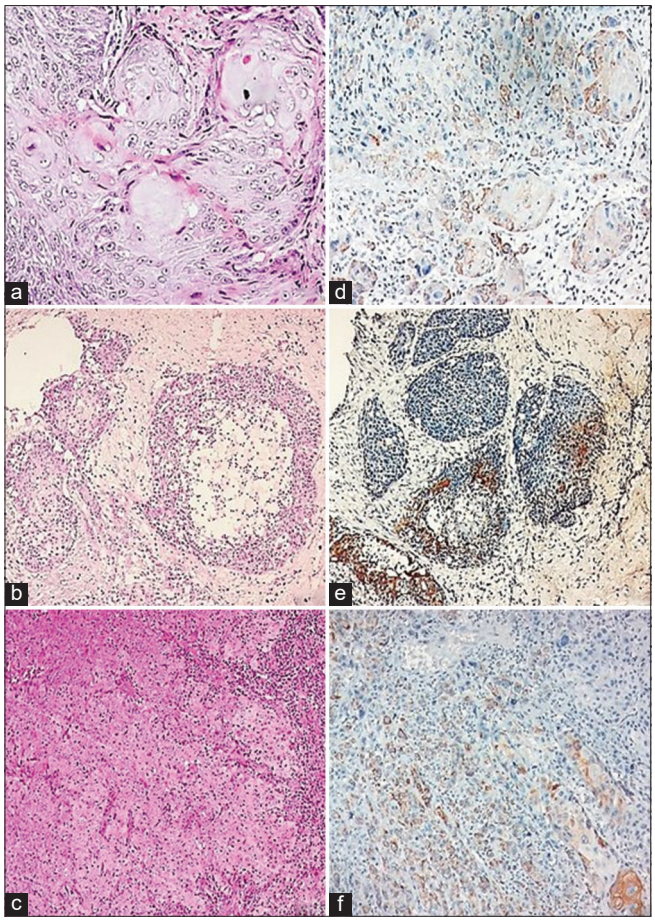

Figure 9: CK19 expression in oral squamous cell carcinoma. (a-c), (a) Well differentiated oral squamous cell carcinoma, (b) Moderately differentiated oral squamous cell carcinoma, (c) Poorly differentiated oral squamous cell carcinoma, H and E, ×10. (d-f) CK19 expression in increasing grades of oral squamous cell carcinoma, IHC, ×10

and the less differentiated islands without keratin pearls, these two were evaluated separately, keeping 30% as the cutoff value of keratin pearls. An increased expression of CK19 was noticed in islands >30% keratin pearls, suggesting an increased expression of CK19 with differentiation. In the case of invading islands of moderately differentiated OSCC, a diffuse scattered expression was noticed with a strong positive expression in some malignant epithelial islands situated superficial in the connective tissue stroma. In poorly differentiated OSCC, invaded epithelial cells distributed in the form of nests, islands and sheets showed a uniform strong cytoplasmic positivity in eight samples of this group [Figure 10]. The mean Allred score was detected to be 2.50 in Group IV.

Comparison of mean Allred scores of well differentiated, moderately differentiated and poorly differentiated OSCC is depicted in Table 4. Kruskal–Wally analysis was applied for statistical analysis and the difference was found to be statistically insignificant with the value of $P = 0.244$. A bar diagram showing a comparison of mean Allred score among different grades of OSCC is shown in Diagram 2.

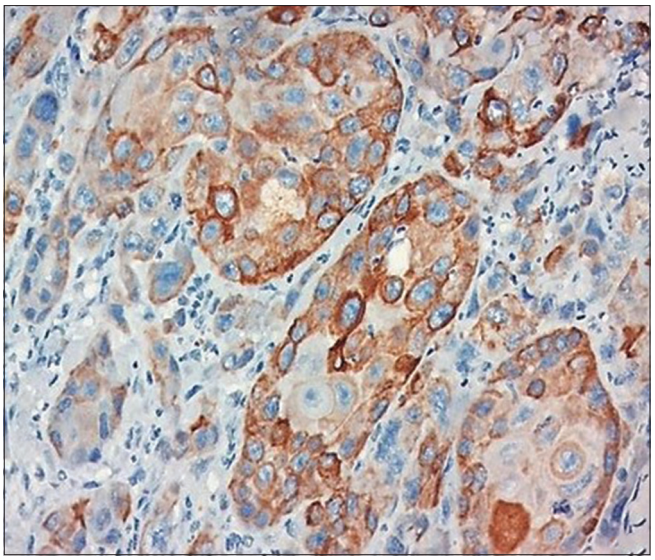

Figure 10: Strong cytoplasmic positivity CK19 noticed in malignant epithelail cells distributed as nests and islands in poorly differentiated oral squamous cell carcinoma, IHC, ×20

DISCUSSION

OSCC still remains a major global health problem, with an overall 5-year survival that has remained at 50%.[5] Hence, a reliable tumor marker can be useful in the early detection of OSCC and monitoring the response to therapy. CK19 is an exceptionally unique type I cytokeratin that is being studied extensively as a differentiation marker, stem cell marker, premalignant, malignant and metastatic marker, as well as diagnostic and prognostic marker too.

In our study, a positive CK19 expression restricted to the basal layer of the epithelium was noticed in both keratinized and nonkeratinized oral mucosa. But according to the literature, most of the studies conducted have reported a null expression of CK19 in the normal keratinized epithelium[6,7] and a positive CK19 expression involving the basal layer in nonkeratinized epithelium.[6,8] A study conducted by Khanom *et al*.[9] in tissue samples obtained from the dorsum of normal tongue documented a basal layer expression of CK19. In samples of epithelial hyperplasia studied, an increased expression of CK19 was noticed with the cells in basal, parabasal, spinous and superficial cells showing a positive expression. A similar expression of CK19 was noticed in the superficial epithelium of focal epithelial hyperplasias associated with inflammatory areas in normal oral epithelium. There existed a direct proportionality between CK19 expression and inflammation and increased expression was noticed with an increase in grades of inflammation. Our findings were in accordance with Ouhayoun *et al*.[10] who

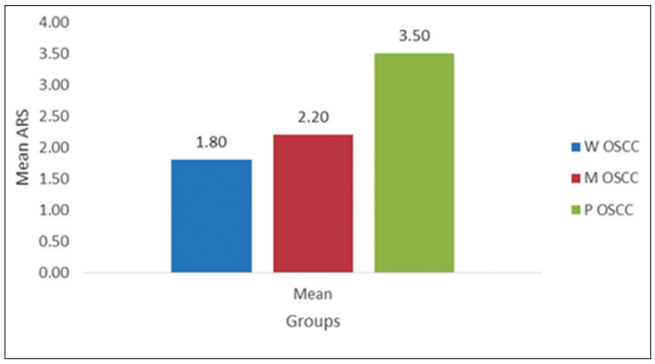

Diagram 2: Comparison among grades of oral squamous cell carcinoma

conducted studies in inflamed gingival hyperplastic lesions reported that an increased CK19 expression is noticed with positive expression seen in cells of basal, parabasal and some spinous cells. A significant correlation between the amount of suprabasal staining of CK19 and degree of inflammation was documented by Bosch *et al*.[11] in inflamed gingival samples. However, contrary to our finding, Coltrera *et al*.[12] found CK19 expression only in the basal layer, if at all present, in hyperplastic lesions. This was supported by Lindberg *et al*.[5] who reported a similar finding.

In this study, there was decreased expression of CK19 in mild and moderate dysplasia with an abrupt increase in expression in severe dysplasia. A progressive increase was not noted in expression of CK19 with increasing grades of dysplasia. Many previous studies have documented a progressive increase in expression of CK19 from mild-to-moderate and severe dysplasia.[6,8,13] Safadi *et al*.[13] documented a progressive increase in expression of CK19 with increasing grades of dysplasia in samples obtained from different grades of dysplasia using an automated color deconvolution program. However, their study was not conducted in an equal number of samples in different grades of dysplasia (23 mild, 8 moderate and 12 severe). An immunohistochemical study performed by Yoshida *et al*.[14] categorized the lesions as low-grade and high-grade dysplasias and compared these with the expression of OSCC samples. They found that a progressive increase in expression was noticed from low grade to high grade and finally OSCC. However, this study could not characterize the difference in expression of CK19 in mild and moderate dysplasia as these were clubbed together in one group. Our study findings are in accordance with the previous findings of Coltrera *et al*.[12] who conducted an immunohistochemical study using two different clones of CK19 in samples from the normal oral cavity and found that CK19 is a specific marker of moderate to severe dysplasia, but this cannot be used as a specific and sensitive marker to distinguish dysplasia from oral epithelial hyperplasia. Marcel *et al*.[15] in his cytological preparation obtained from samples of tongue, carcinoma concluded that CK19 expression is a marker related to premalignancy. However, contradictory findings were reported by Coltrera *et al*.[12] who postulated that increased CK19 expression in tissue samples with inflammation may be associated with a metaplastic rather than premalignant change.

A decrease in expression of CK19 was seen in superficial epithelium of well to moderate and poorly differentiated OSCC with positive reaction interspersed in cells of basal and parabasal layers. However in areas of invasion, increased expression was observed, with CK19 positivity involving the cells of basal, parabasal and spinous layer. A similar finding was reported by Sawant *et al*.[16] and Khanom *et al*.[9] and found that CK19 positive cancers showed a more invasive tumor front than the CK19 negative cancers. The underlying mechanism remains unclear. However, according to Crow *et al*.,[17] there is a decreased expression of CK19 in superficial epithelium showing an invasive potential. A diffuse scattered expression of CK19 with increasing grades of OSCC, as seen in our study, has been reported by Safadi *et al*.[13] Literature findings indicate that the opinion on CK19 expression in OSCC remains divided. A decreased expression of CK19 has been reported in samples of well-differentiated OSCC when compared to higher grades of carcinoma.[13,17] Zhong *et al*.[18] found a significant correlation between CK19 expression and pathologic differentiation grade using immunohistochemistry. Similarly, a progressive increase in expression of CK19 with increasing grades of carcinoma has been reported by Safadi *et al*.,[13] Fillies *et al*.[19] and Ram Prasad *et al*.[20] in their individual studies. However, according to Crow *et al*.,[17] there is a decrease in expression of CK19 in higher grades of invasive carcinomas. This was supported by Kobayashi *et al*.[21] and Khanom *et al*.[9] in their respective studies. However, many studies in the literature suggests that overexpression of CK19 in OSCC samples has been detected to show a poor prognosis.[17-19] In this study, there was a progressive increase in expression of CK19 among different grades of OSCC, but the difference noted was not statistically relevant.

SUMMARY AND CONCLUSION

1. In normal oral epithelium, basal expression of CK19 was noticed in tissues from keratinized as well as non-keratinized mucosa which usually do not express CK19
2. An increased expression of CK19 was noticed in areas of inflammation, suggesting the role of inflammatory cytokines produced by the chronic inflammatory cells in the subepithelial connective tissue in inducing the expression of CK19 in the overlying epithelium
3. Our findings showed that increased expression of CK19

occurs with hyperplasia and severe dysplasia. In case of mild and moderate dysplasias, CK19 expression was found to be lower than that of normal mucosa. However, an abrupt increase is noticed in severe dysplasia, suggesting that during the initial phase of dysplasia due to some reason, the expression decreases and as the lesion progresses to the advanced stages of dysplasia, the expression increases. A similar increased expression was also noticed in the superficial epithelium at the invading front in OSCC. A progressive increase in expression with progression of dysplasia was not noticed in the present study. From these findings, we may conclude that CK19 expression is induced in later stages of progression in epithelial dysplasias

4. In OSCC, the expression of CK19 was restricted to either a few islands or a few cells within the islands, thereby bringing CK19 expression to a value less than what is noticed in normal epithelium. The malignant epithelial islands in the superficial connective tissue stroma were showing greater expression than the deeper islands and those epithelial cells associated with the formation of keratin pearls were found to be showing more expression than those with infrequent keratin pearls
5. Our study questions the use of CK19 as a single stem cell marker in the diagnosis and prognosis of oral cancer as it is found to be expressed only in certain phases of progression
6. Although it cannot be used as a progression marker, as a significant increase in expression is noticed in severe dysplasias and invading the front of the superficial epithelium in OSCCs, it appears that malignant transformation of epithelium can be predicted based on the increased expression of CK19. However, it should be done with caution as a similar increased expression can be noticed in the presence of inflammation too.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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