Immunohistochemical Expression of Ck8 and Ck18 in Oral Potentially Malignant Disorders and Oral Squamous Cell Carcinoma - A Retrospective Study On 70 Samples

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Abstract

Background: Oral squamous cell carcinoma (OSCC) prevalence is high in India and contributes to about 90% of all oral neoplasms. The 5 year survival rate has not improved even with advances in treatment options. It could be due to delay in patient reporting and lack of proper histopathological examination. Immunohistochemistry helps to confirm the diagnosis in suspicious cases promptly.

Aim and Objectives: Aim of the present study is to identify variations in the immunohistochemical expression of CK 8/18 in normal mucosae, oral potentially malignant disorders (OPMD) and oral squamous cell carcinoma (OSCC).

Materials and Methods: A total of 70 samples (Formalin Fixed Paraffin embedded blocks) including 10 normal oral mucosa, 30 OPMD and 30 OSCC histologically diagnosed cases were included in the study. Immunohistochemical staining of CK8 and CK18 was performed. The color grading and assessment of IHC staining of the slides was done by two oral pathologists . The data were tabulated and analyzed with SPSS v 22.0 (SPSS Inc, Chicago, IL) using Mean ± SD, ANOVA, Chi Square test Tukey HSD Post-hoc Test. A probability of $p<0.05$ was considered as significant.

Results: The mean score in the control group was 0.9 with standard deviation 1.1. The mean score in the OPMD group was 1.13 with standard deviation 0.8. The mean score observed in the OSCC group was 1.45 with standard deviation 0.86. We performed the Tukey HSD post-hoc test and evaluated that Difference between control and OPMD group was 0.2300, and p value is 0.7606, which did not achieve statistical significance. Difference between Control and OSCC was 0.5500. p value 0.2169, which was not significant. Differences between OPMD and OSCC group was 0.3200 with p value was 0.3520 which also not significant.

Conclusion: Strong expression was noted in OPMD and OSCC in our study. There was a progressive increase in its expression from the normal mucosa to OPMD to OSCC but our values did not reach statistical significance. Further investigations will be necessary to explore the mechanisms of CK8/18 expression as well as their exact functions in such cancerous lesions to improve our understanding on tumor biology and its behavior.

Keywords: Oral squamous cell carcinoma (OSCC), oral potentially malignant disorders (OPMD), immunohistochemical staining (IHC), Cytokeratin 8 (CK8), Cytokeratin 18 (CK18).

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INTRODUCTION

Cytokeratins (CK) are the principal intermediate filament proteins of epithelial cells. Its expression is noted in different malignancies and is associated with patient prognosis. 1,2,3

Depending on their tissue expression pattern, they have been grouped into simple epithelia specific CK (CK7, 8, 18, 19, 20) and stratified epithelia specific CK (CK 4, 5, 13, 14 etc). The most abundant epithelial CK are CK8, 18 and 19.15 CK18 and CK19 are observed in salivary epithelium. CK 7, 8, 18, 19 are identified in luminal duct cells. CK 8 and CK18 observed in

epithelium of striated and intercalated ducts.Oral leukoplakia and oral submucous fibrosis are two major known OPMDs and 8-10% is the malignant transformation rate. Newer methods are needed to predict the malignant potential of OPMD and determine the prognostic value. Immunohistochemical evaluation helps in categorizing such OPMD and to predict locoregional recurrence and regional lymph node metastasis. 4-15

Studies reveal that low molecular weight CK 8, 18 were expressed in poorly differentiated hypoxic variants of OSCC with higher degree of cell cycle deregulation than well differentiated variants of OSCC showing high molecular weight CK like 1, 5, 6, 10 and 14. 16-19

Cytokeratins demonstrate a specific expression pattern which is site- specific and varies with the level of differentiation. This property of cytokeratin has evolved as a potential epithelial differentiation marker in cell biology, embryology and surgical pathology. Cytokeratins are the 'gold standard markers' in immunohistochemical diagnosis, classification and subtyping of carcinomas and detection of unclear metastasis. Soluble cytokeratin protein fragments detection is recently adopted as a tool to check tumor load and prognosis of carcinomas.20-21 Along with the expression of CK 8/18 and CK 19, noted that over expression of MMP-9 was seen in all cases of OSCC predicting invasive tumor progression. 22-23 Recently, it is been stated that cytokeratin expression is not restricted to carcinomas, few sarcomas with true epithelial differentiation (e.g.: synovial and epithelioid sarcomas) also are positive for specific cytokeratin types.24

CK8 and CK18 are described as surrogate markers of malignant transformation in a squamous epithelium. CK8/18 is the first pair expressed in the process of embryogenesis. CK8/18 is unique in many ways. All type I CK genes are located on chromosome 17, while all type II CK genes are situated on chromosome 12. Although CK8 and 18 belong to the type II and type I subfamilies, respectively, both are found on chromosome 12. CK8 and 18 are normally expressed by simple epithelia, while stratified epithelia do not express this pair. SCCs derived from stratified epithelia show aberrant expression of these Keratins.15, 25-34

Cytokeratin deposition has been reported to occur in the necrotic region intratumorally because of increased proteolytic activity in these cells. The possibility of using these deposited fragments of CK as stable targets for radioimmuno-localization and radioimmuno-therapy of some cancer is being investigated. Another consequence of the increased proteolytic activity in tumor cells is the appearance of CK fragments in the sera of cancer patients.22 Cytokeratin 8–18 expression in OSCC harbors a significant prognostic potential. Results of many studies show that modulating the expression of an intermediate filament protein results in sensitization to chemotherapeutic drugs. The subgroup of CK 8–18 positive squamous cell carcinoma may have a benefit by chemotherapy with cisplatin. Further investigations are necessary to determine the clinical impact of these hypotheses. Earlier results on CK expression in OPMD and OSCC have shown two types of changes like Aberrant expression of some CK like CK 1, 8, 16, 17 and 18 and Non-expression of certain normally expressed CK like CK 5. 15

To explore the possibility of detecting these alterations in CK 8 and 18, a retrospective study was conducted on OPMD and OSCC for the expression of CK 8 and18 using immunohistochemistry.

Aims And Objectives Of The Study

To determine the expression of CK8 & CK18 in potentially malignant oral disorders.

To determine the expression of CK8 & CK18 in oral squamous cell carcinoma.

To compare the expression of CK8 & CK18 in oral potentially malignant disorder and oral squamous cell carcinoma.

Materials & Methods

The present study was divided under following divisions

Collection of OPMD and OSCC samples

Immunohistochemical staining with CK 8 and CK 18

Evaluation of slides and scoring

Statistical analysis of the data

OPMD and OSCC cases from the Archives of Department of Oral and Maxillofacial Pathology and Oral Microbiology, Surendera Dental College and Research Institute, Sriganganagar, Rajasthan were retrieved.

The inclusion criteria were Histopathologically diagnosed cases of OPMD and OSCC with tissue blocks of adequate size (minimum of 5mm). The exclusion criteria were OPMD of sites other than oral cavity proper like oropharynx, maxillary sinus etc, Carcinomas of sites other than oral cavity proper like oropharynx, maxillary sinus etc and Tissue blocks without adequate size (less than 5 mm).

A total of 70 samples including 10 normal oral mucosa, 30 OPMD and 30 OSCC cases were included in the study. Formalin fixed paraffin embedded tissue sections was considered for immunohistochemical analysis using monoclonal antibodies to CK 8/18 (Polymer IHC Detection Kit (Biogenex; CA, USA) using EZ Retriever system V.2.1, Biogenex; USA. 3µ sections were made with a Yorco YSI-062 Fully automatic rotary microtome. It was stained routinely with Hematoxylin and Eosin as per manufacturer instructions. The stained slides were reevaluated and reconfirmed for the histopathological diagnosis of OPMD and OSCC by 2 independent oral pathologists. Immunohistochemical staining for CK 8/18 was performed with Primary Antibody: Anti-Cytokeratin 8 & 18 Monoclonal (AM131-5M), Biogenex; CA, USA. Chromogen: Liquid DAB (3.3'-diaminobenzidine) chromogen solution; Biogenex; CA, USA. For each batch of staining, a positive control and a negative control was used by replacing the primary antibody with a negative control buffer.

The stained slides were interpreted by two oral pathologists for positivity shown as brown staining due to DAB chromogen. H and E stained slides were used for comparison of IHC stained slides results. The fields containing artifactual changes such as chatter, tears etc were omitted from the study. Any non-specific positivity was not taken into account. The slides were analysed using the binocular Labomed research microscope (LX400), digital camera, Desktop or a Laptop computer, PIXELPRO software (LABOMED Inc, USA) and DIGIMER image analysis software (Medcalc software BVBA, Belgium)

Quantitative evaluation of CK 8 & 18 positive cells

Briefly, in an optical microscope, hot-spot areas for CK 8 & 18 expression within epithelial cells were initially identified by scanning the entire section in low power (10x). Based on the hotspot areas under low power, the number of CK 8 & 18 positive cells in five of these areas was then counted in high power magnification (40x) using the binocular microscope attached to a computer with image analysis software. Presence of brown colored end product at the site of target antigen was indicative of positive reactivity. The negative control does not show specific staining. The positive control used here was salivary glands. Any stromal positivity for CK 8 & 18 was not taken into account.

The number of CK 8 & 18 positive cells was counted in 50 cells of each field by two observers. The scoring was as follows: (-) no color, (+) Yellow, (++) Light brown, and (+++) Dark brown. Cases were assigned to one of the following categories: 0% positive cells (-), 10% positive cells (+), 10-25% positive cells (++), 26-50% positive cells (+++) or more then 50% positive cells (++++).

Statistical Method

The data were tabulated and analyzed using Mean ± SD, ANOVA, Chi Square test, Tukey HSD Post-hoc Test. A probability of $p<0.05$ was considered as significant.

Statistical Software

SPSS v 22.0 (SPSS Inc, Chicago, IL) was used for statistical analysis.

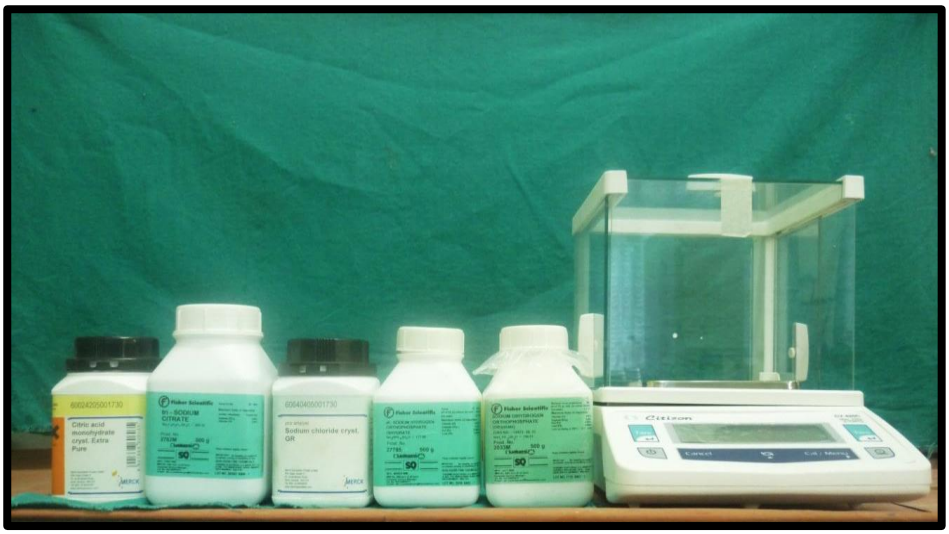


Fig. 1: Reagents for preparing stock solutions for immunohistochemistry and weighing balance

Fig. 2: Stock solutions (antigen retrieval and buffer)

Fig. 3: Working Solutions (Antigen Retrieval and Phosphate Buffer Saline)

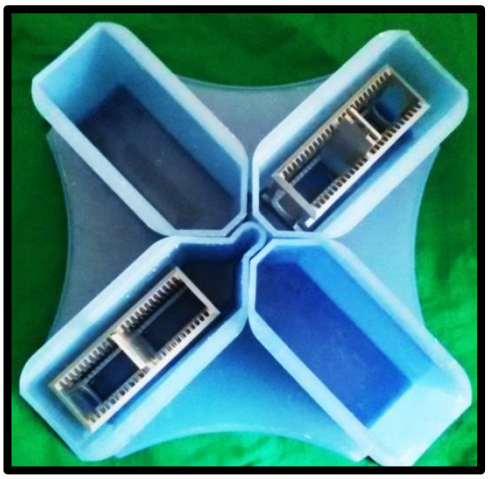
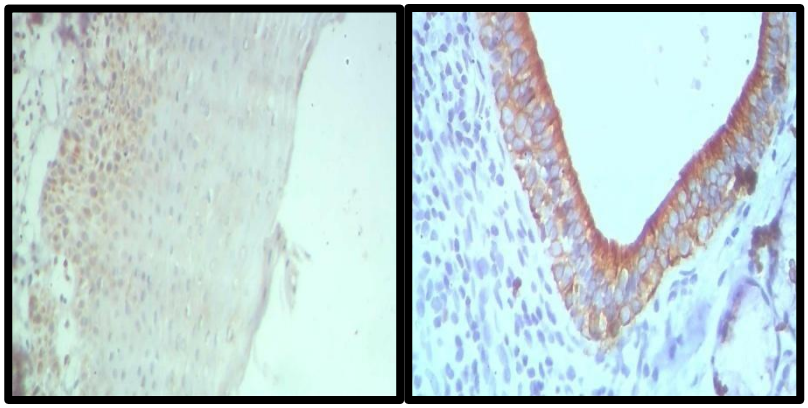


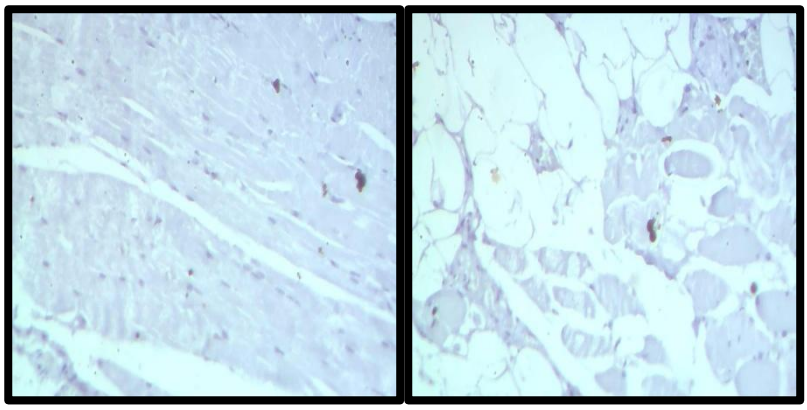
Fig. 4: Antigen retrieval system (Biogenex)

Fig. 5: Polymer kit (super sensitive polymer – HRP- IHC detection kit)

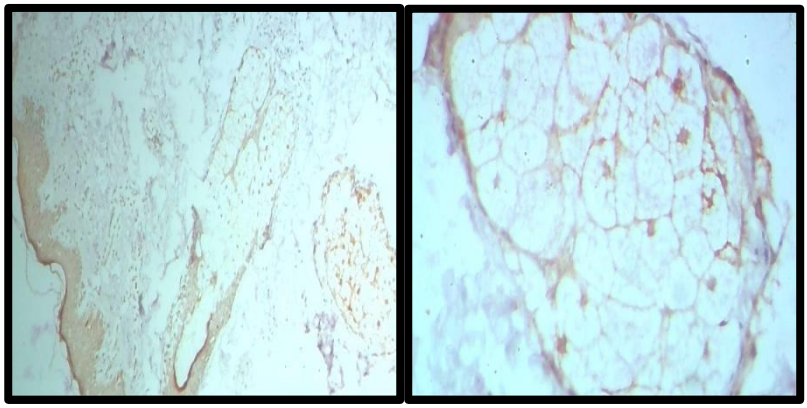
Positive Control Group



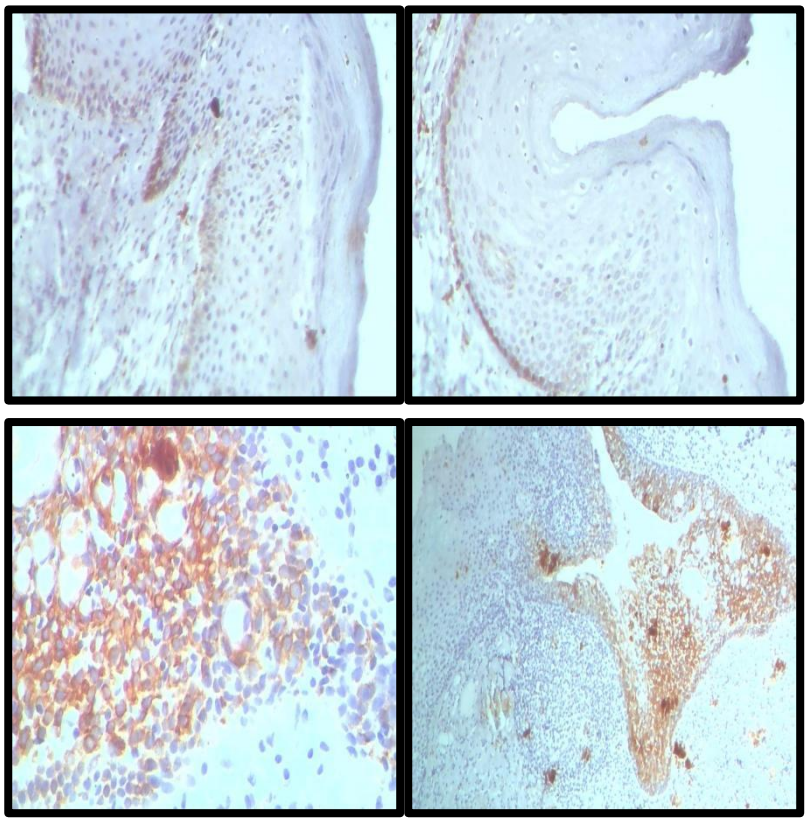
Negative Control Group



Skin sample with its accessory structures



OPMD group



OSCC Group

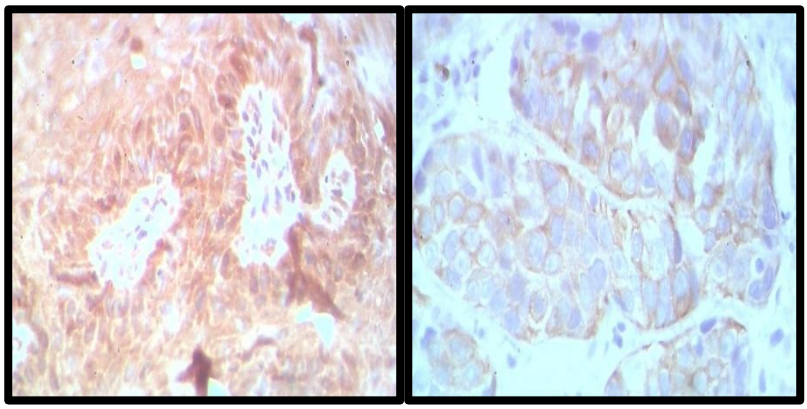


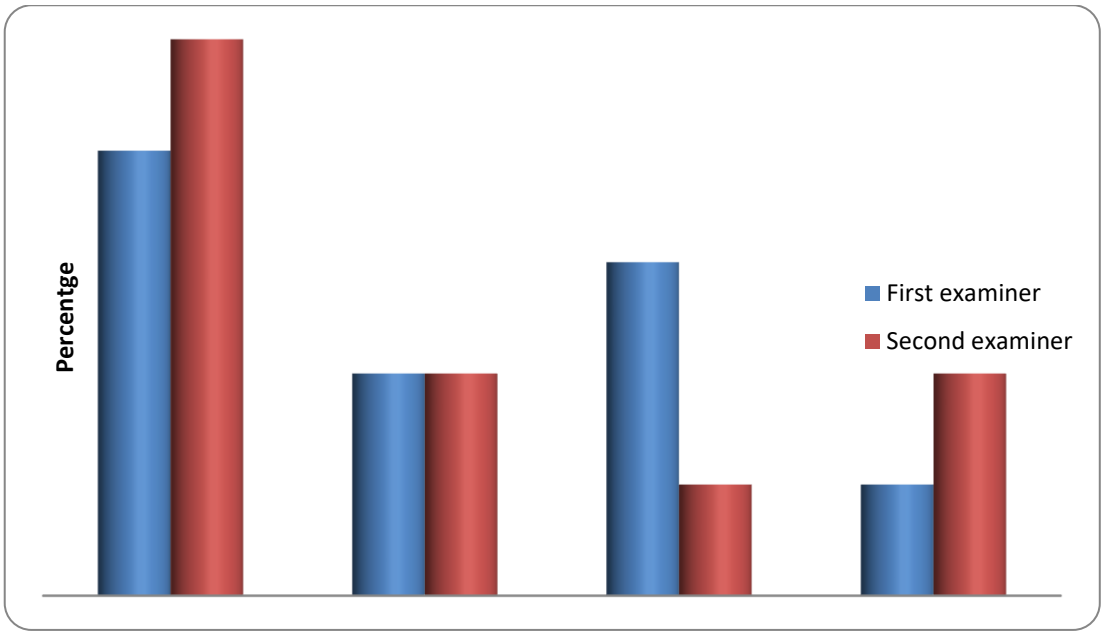
Figure 6 - Photomicrographs of Immunohistochemical staining (CK 8/18 - 10x)

Results

Table 1: Table showing the grading of color and CK8/18 positivity among the control group.

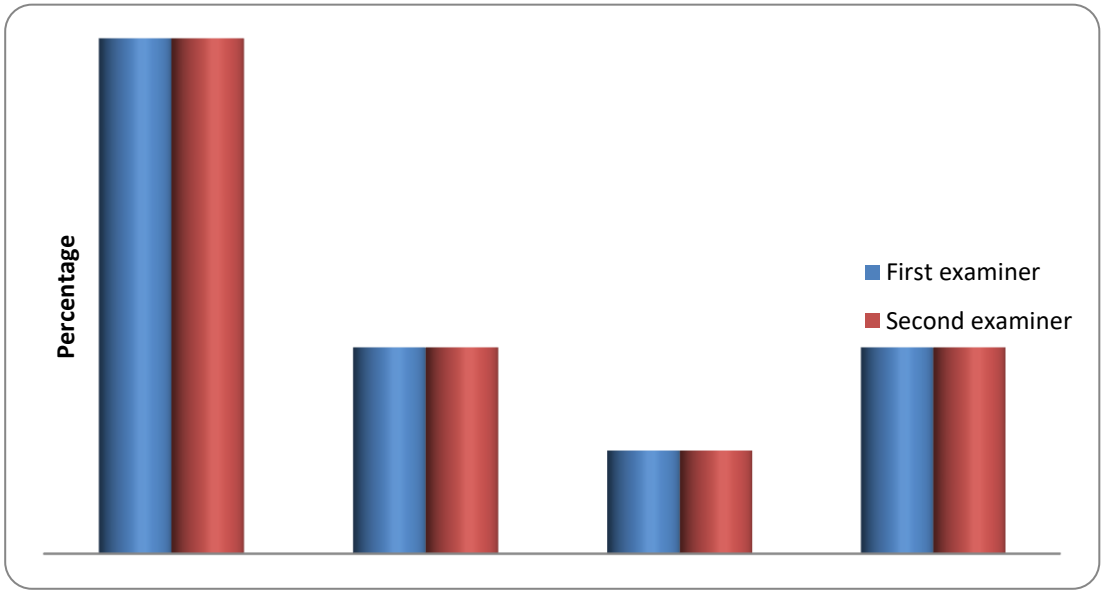
Grading system	First examiner		Second examiner	
	N	%	N	%
Colour Grading				
No colour=0	4	40	5	50
Yellow=1	2	20	2	20
Light Brown=2	3	30	1	10
Dark Brown=3	1	10	2	20
Chi square	1.44			
p value	0.69			
Grading of CK8/18				
0 (0% +ve cells)	5	50	5	50
1 (10% +ve cells)	2	20	2	20
2 (10-25% +ve cells)	2	20	2	20
3 (26-50% +ve cells)	1	10	1	10
Chi square	0			
p value	1			

Table 1 shows the number of cases and their graded colors along with the percentage given by two oral pathologists among the control group. It also shows the grading of cells among the control group. It was observed that out of 10 samples 4 (40%) by 1st observer and 5(50%) samples by 2nd observer was not showing any colour, 2 (20%) samples were showing yellow colour by both observers. It was observed that 3(10%) and 1(10%) value for light brown, dark brown colour values were 1(10%) and 2(20%) respectively. Grading for number of positive cells showed same values by both the observers as follows, 50% of samples were negative (-), 20% of samples were (+), 20% of samples were (++) and one sample showed (+++) staining.



Graph 1: Color grading among control group

Graph 1 shows the graded colours along with the percentage of control group as scored by two oral pathologists.



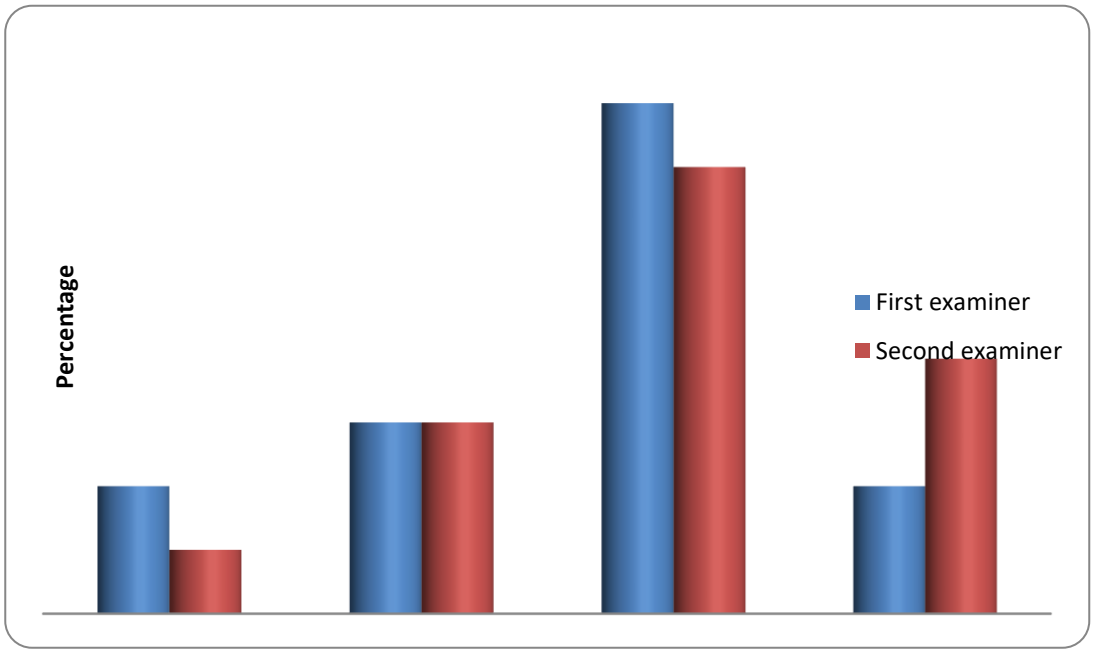
Graph 2: control group

Graph 2: shows the percentages of positive cells among the control group as scored by two oral pathologists.

Table 2: Table showing the grading of color and CK8/18 positivity among the Potentially Malignant.

Grading system	First examiner		Second examiner	
	N	%	N	%
Color Grading				
No color=0	4	13.33	2	6.67
Yellow=1	6	20	6	20
Light Brown=2	16	53.33	14	46.67
Dark Brown=3	4	13.33	8	26.67
Chi square	2.13			
p value	0.55			
Grading				
0 (0% +ve cells)	9	30	5	16.67
1 (10% +ve cells)	12	40	15	50
2 (10-25% +ve cells)	8	26.67	8	26.67
3 (26-50% +ve cells)	1	3.33	2	6.67
Chi square	1.81			
p value	0.61			

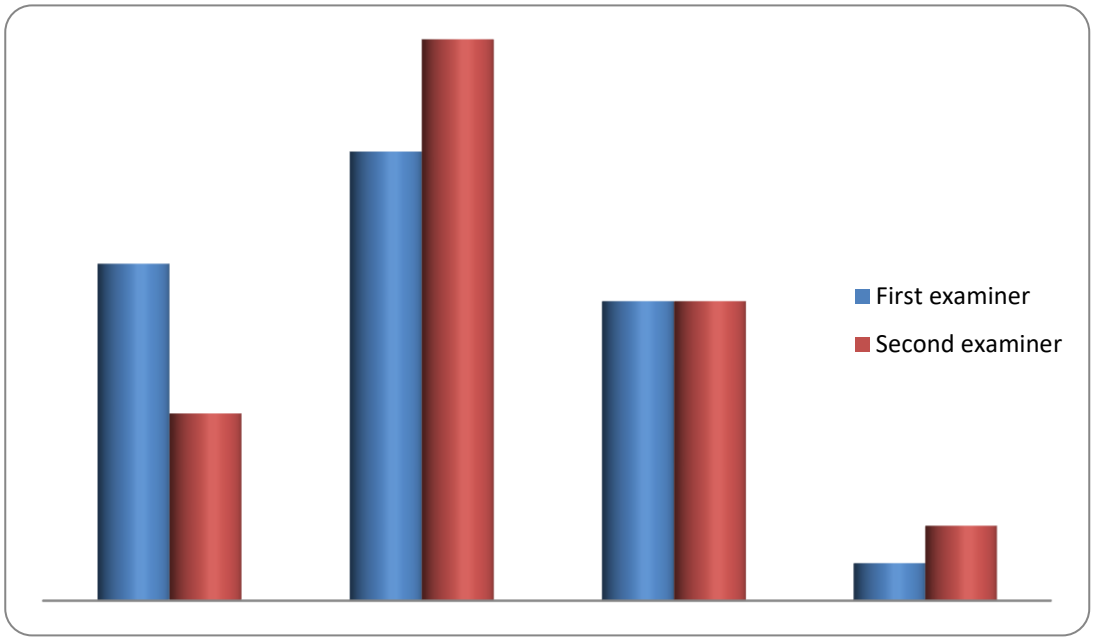
Table 2 shows the number of cases and their graded colours along with the percentage given by two oral pathologists among Potentially Malignant group. It also shows the grading of positive cells among the Potentially Malignant group. It was observed that out of 30 samples 4 (13.33%) by 1st observer and 2(6.67%) samples by 2nd observer showing negative colour, 6 (20%) samples were showing yellow colour by both observers. It was observed that 16(53.33%) and 14(46.67%) values for light brown and 4(13.33%) and 8(26.67%) for dark brown by both observer respectively. Out of 30 samples, 9(30%) and 5(16.67%) scored (-), and 12 (40%) and 15(50%) scored (+), respectively. By both observers (++) scored given to 8 (26.67%) samples. 1(3.33%) and 2(6.67%), positive cells scored (+++) respectively by both observer.



Graph 3: Potentially Malignant group

Graph 3 shows the colours along with the percentage given as scored by two oral pathologists among the Potentially Malignant

group.



Graph 4: Potentially Malignant group

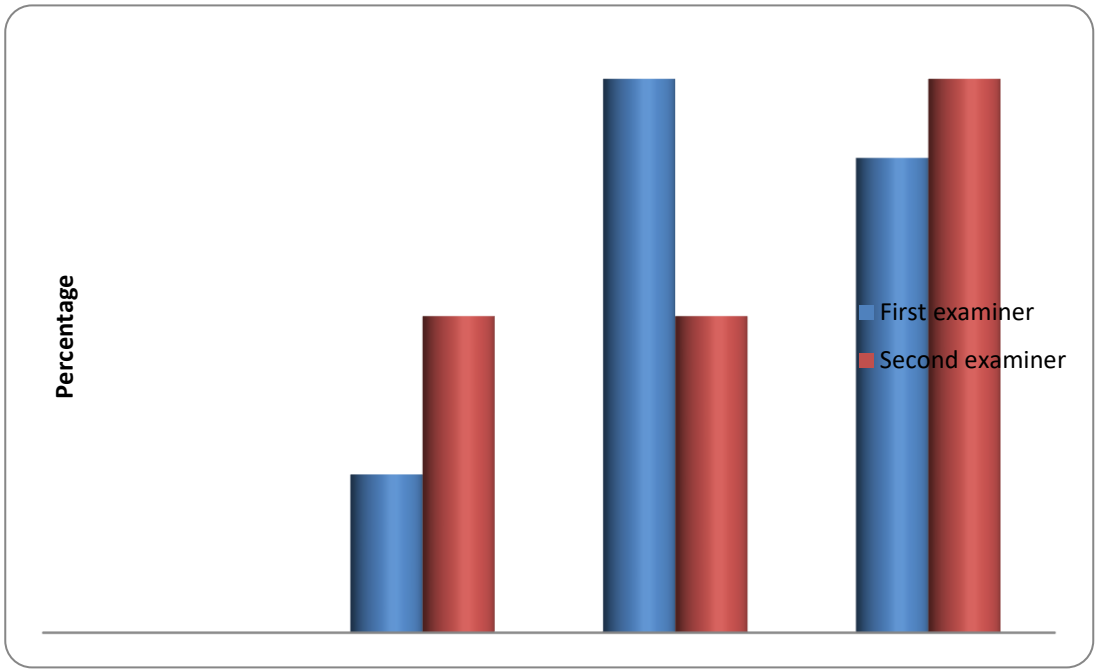
Graph 4 shows the percentages of positive cells among the potentially malignant group as scored by two pathologists.

Table 3: Table showing the grading of color and CK8/18 positivity among the OSCC group.

Grading system	First examiner		Second examiner	
	N	%	N	%
Color Grading				
No color=0	0	0	0	0
Yellow=1	4	13.33	8	26.67
Light Brown=2	14	46.67	8	26.67
Dark Brown=3	12	40	14	46.67
Chi square	3.12			
p value	0.37			
Grading				
0 (0% +ve cells)	2	6.67	3	10
1 (10% +ve cells)	17	56.67	17	56.67
2 (10-25% +ve cells)	6	20	4	13.33
3 (26-50% +ve cells)	5	16.67	6	20
Chi square	0.69			
p value	0.87			

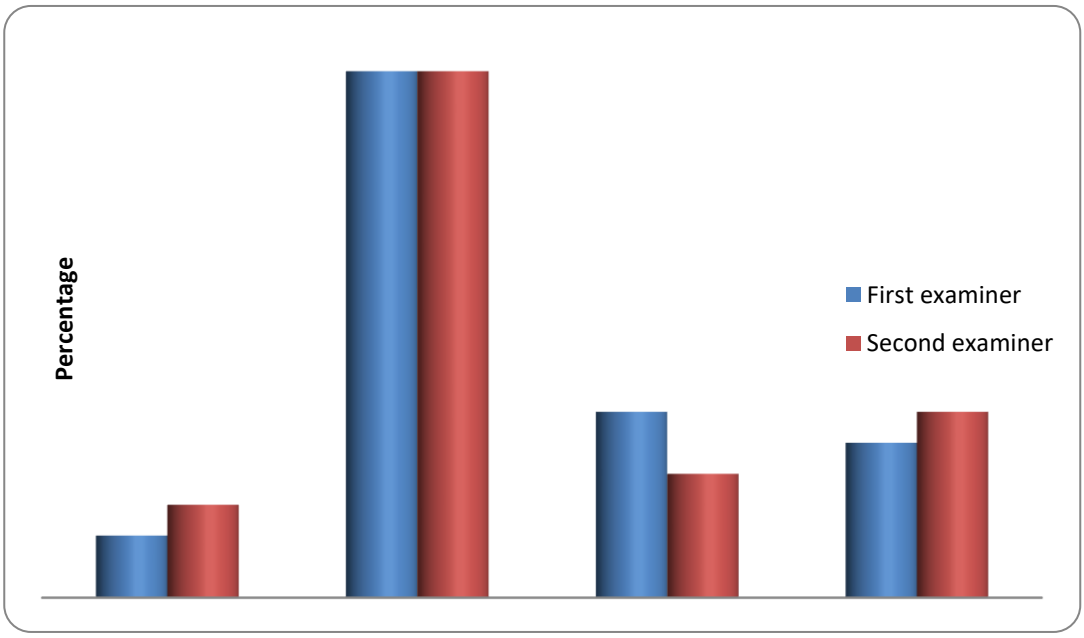
Table 3 shows the number of cases and their graded colours along with the percentage given by two oral pathologists among Cancer group. Both the observers were found that all the samples taking color. According to 1st observer 4(13.33%) and according to 2nd observer 8(26.67%) samples were taking the yellow color. 14(46.67%) and 8(26.67%) were taking the light brown color and 12(40%) and 14(46.67%) samples showing the dark brown respectively. 2(6.67%) and 3(10%) scored (-) by both observers respectively. Both the observer scored (+) to 17(56.67%) samples, 6(20%) and 4(13,33%) scored the (++),

whereas 5(16.67%) and 6(20%) scored the (+++).



Graph 5: OSCC group

Graph 5 shows the colour of immunohistochemical staining along with the percentage as scored by two oral pathologists among the OSCC group.



Graph 6: OSCC group

Graph 6 shows the percentages of positive cells among the OSCC group as scored by two oral pathologists.

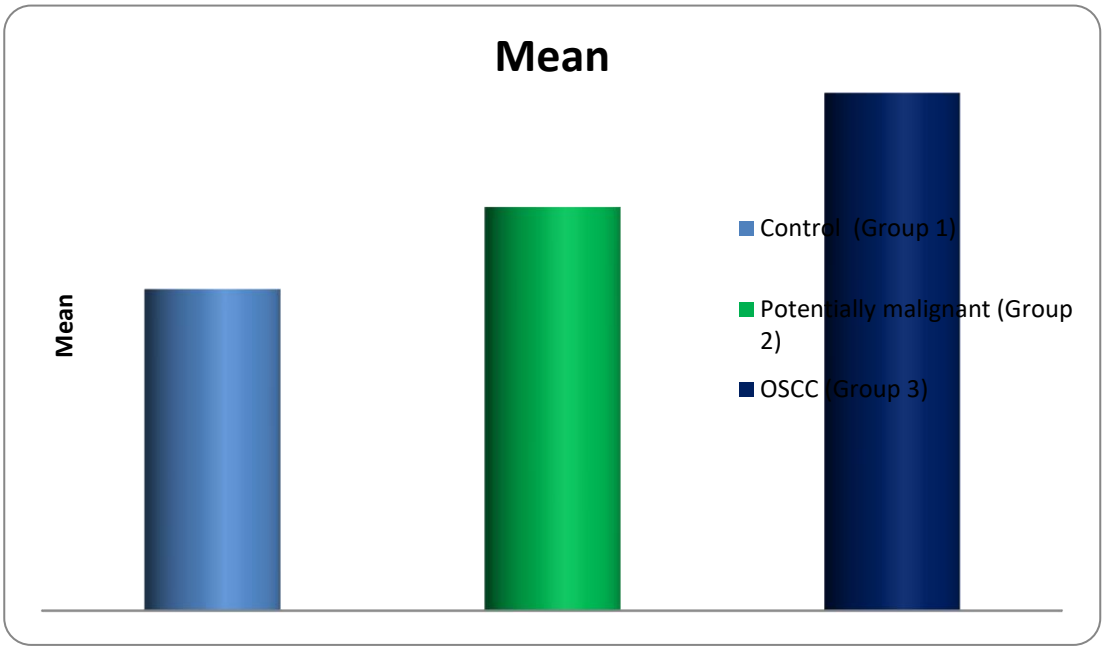
Table 4: Mean grading scores

Groups	Mean±SD	Anova test	p value
Control (Group 1)	0.9±1.1	1.79	0.18
Potentially malignant(Group 2)	1.13±0.85		
OSCC (Group 3)	1.45±0.86		
Tukey HSD Post-hoc Test... Control vs Potentially Malignant: Diff=0.2300, 95%CI=-0.5506 to 1.0106, p=0.7606 Control vs Cancer: Diff=0.5500, 95%CI=-0.2306 to 1.3306, p=0.2169 Potentially Malignant vs Cancer: Diff=0.3200, 95%CI=-0.2319 to 0.8719, p=0.3520			

All p values are non-significant

Table 4 shows the mean scores of controls, potentially malignant and cancer group. It also shows the comparisons between the study groups. The mean score in control group was 0.9 with standard deviation 1.1. The mean score in the potentially malignant group was 1.13 with standard deviation 0.8. The mean score observed in OSCC group was 1.45 with standard deviation 0.86.

We performed the Tukey HSD post-hoc test and evaluated that Difference between control and Potentially Malignant disorders was is 0.2300, and p value is 0.7606, which did not achieve the statistical significance. Difference between Control and OSCC group was 0.5500. p value 0.2169, which was not significant. Differences between Potentially Malignant disorders and OSCC group was 0.3200 with p value was 0.3520 which also not significant. All the p values did not achieve the statistical significance.



Graph 7: Mean comparison of grading scores among the three groups

Graph 7 shows the Mean values of the scores for cytokeratin 8/18 expression between the study groups shows that Control< PMD<OSCC.

Discussion

According to BE Sundström and TI Stigbrand (1994), Cytokeratins 8, 18 and 19 are the most abundant cytokeratins in carcinomas. They are released into necrotic areas and can be found intratumorally and in blood, circulating as partially degraded complexes, and can as such be used as tumor markers.

This retrospective study was carried out in the Department of Oral & Maxillofacial Pathology and Oral Microbiology, Surendera Dental College and Research Institute, Sriganganagar, Rajasthan, India on Paraffin embedded blocks of histologically diagnosed cases of Normal Oral Mucosa (N=10), Oral potentially malignant disorders (OPMD=30) and Oral squamous cell carcinoma (OSCC=30). The study consisted of Immunohistochemical expression of CK 8 & 18 in Normal Oral Mucosa, OPMD and OSCC.

CK 8 & 18 monoclonal antibody from Biogenex; CA, USA was used for analysis. Similarly, Ranganathan et al,52 Thomas F, JOGSCHIES M et al3, Kittipong Dhanuthan et el54, Matthias C et al,53 Rao V et al2 in 2008, Alka D Kale et al23 , Nanda et al27 in 2012, Mitra S.K. et al35 had performed the study with the same antibody.

Fillies T et al 3, Frohwitter G et 19 al had performed the study by a standard avidin-biotin complex method with a biotinylated rabbit anti-mouse antibody (DAKO) and an avidin-biotin complex (DAKO). Similarly, Afrem et al had performed the study with the LSAB (Labeled Strepatavidin-Biotin 2 System) technique. The kit used was manufactured by Dako, Redox, Romania (Code K0675). Similarly, Ogden GR et al48 followed the Vectastain ABC method as their immunocytochemical technique.

The present study comprised a total retrieved 70 Paraffin embedded blocks of histologically diagnosed samples, 10 of Normal Oral mucosa and 30 OPMD and 30 OSCC. Out of the 30 OPMD, 10 were mild dysplasia and 20 were moderate dysplasia. Similarly, Ogden G R et al in studied on 24 oral cancer and 15 normal oral mucosal samples, Ranganathan et al52 studied 50 oral submucous fibrosis samples, Thomas F et al3 studied on 180 OSCC and 13 oral leukoplakia and dysplasia samples, Kittipong Dhanuthan et el54 studied 30 lichen planus samples, Thomas et al studied on 192 OSCC, 117 patients with oral leukoplakia without dysplasia (OL) and 23 oral leukoplakia with dysplasia (OLD) samples, Matthias C et al53 studied 60 OSCC samples, Sawant S et al15 studied 62 leukoplakia samples, Makino T et al47 studied on 210 oesophageal SCC samples, Alka D Kale et al23 studied on 20 OSCC and 10 normal mucosa samples, Nanda et al studied 10 samples of leukoplakia and 10 samples of oral submucous fibrosis, Mitra S.K. et al35 studied 60 OSCC,40 benign and inflammatory lesions and 4 oral intraepithelial samples, Afrem M.C. et al36 studied on 15 lingual SCC (4 well differentiated, 8 moderately differentiated, 3 poorly differentiated) samples. Frohwitter G et al19 studied 193 oral SCC samples.

In the present study, the percentage of stained cells were estimated in 5 randomised microscopic fields by two oral pathologists and classified as (-): 0%, (+): <10%, (++): 10-25%, (+++): 26-50%, (++++): >50% positive cells .We also graded samples on the basis of color staining (No color=0, Yellow=1, Light Brown=2 and Dark Brown=3). Fillies T et al3 in 2005 determined independently the percentage of positive cells in each core. They classified CK8/18 in two groups (0: no expression, 1:≥1% positive expression). The mean percentage value of the two scores represented one tumor and was used for further evaluation. Ogden GR et al48 graded keratin expression on a 3 point scale. T Makino et al47 determined that presence of CK18 or CK8 protein was positive when the proportion of immunohistochemically stained cells was more than 50% of all observed cancer cells or otherwise negative.

Some other authors used different gradients for the percentage of stained cells. Alka D kale et al23 had three oral pathologists. Seven high-power fields were randomly selected for observation. A section was scored according to the staining intensity and staining area. The staining intensity was scored as, no staining (score 0), light yellow (score 1), yellow to brown (score 2), and dark brown (score 3). The staining area were scored as, no staining (score 0), positive staining for less than one-third of tissue section (score 1), positive staining area ranged from one-third to two-third of tissue section (score 2) and positive staining for more than two-third of tissue section (score 3). Sections were considered negative or positive according to the sum of above two scoring systems and a score ≥3 was regarded as positive. Percentages were calculated to determine the expression of these markers.

Mitra S.K et al35 used positive control for every antibody to eliminate the possibility of wrong interpretation. Cytoplasmic staining was taken as positive in epithelium and number of cells showing positivity was graded as follow- <5% reactive cells= Negative, 5%-30% reactive cells= + (Weak intensity), 30%-60% reactive cells= ++ (Moderate intensity), >60% reactive cells= +++ (Maximum intensity)

Afrem M.C et al36 assess the expression of CK18 was qualitatively performed, the reaction intensity being measured according to the score: 0 – negative, 1 – weakly positive, 2 –moderately positive, 3 – strongly positive. The images were captured using the Nikon Eclipse 55i microscope (Nikon, Apidrag, Bucharest, Romania), equipped with a video camera with 5 megapixel cooling, while the processing and the interpretation were performed with the imaging software AMS7 Image ProPlus (Media Cybernetics Inc., Buckinghamshire, UK). The semiquantitative analysis assessed the number of positive cells at a magnification ×400 on five random fields. The results were grouped as : 0 – absence of reactivity, +1 (weak) – less than 10% of positivity in tumor cells, +2 (moderate) – homogeneous or intense in 10–75% of tumor cells and +3 (intense) – intense homogeneous in more than 75% of tumor cells. Frohwitter G et19 al graded CK8/18 (0%, no expression; ≥1%, positive expression). The mean

percentage value of two cores from one tumor was calculated. Fukuda M et al38 found the positive reaction for CK18 was mainly observed in the luminal columnar cells of adenosquamous carcinoma, whereas it was negative in OSCC.

Vaidiya M.M et al 55 showed that these different CK antigens were expressed in different cell layers. CK 1 were present in the stratified epithelial layers whereas CK 8 and 18 were restricted to glandular epithelium. Till 27 weeks of gestation, both tongue and BM expressed CK 1,8 and 18 along with CK 6 and 16. Thus, foetal tissues showed some similarities in CK pattern with their respective malignant counterparts.

In our study, the mean score in the control group was 0.9 with standard deviation 1.1. The mean score in the OPMD group was 1.13 with standard deviation 0.8. The mean score observed in the OSCC group was 1.45 with standard deviation 0.86. We performed the Tukey HSD post-hoc test and evaluated that the difference between control and OPMD was 0.2300, and p value is 0.7606, which did not achieve statistical significance. Difference between the Control and OSCC group was 0.5500. p value 0.2169, which was not significant. Differences between OPMD and OSCC group was 0.3200 with p value was 0.3520 which also not significant. All the p values did not achieve the statistical significance.

Ranganathan et al 27 found that CK 18 expression was 12% in the examined 50 samples of oral submucous fibrosis (OSF), which inferred that the aberrant expression of CK18 in these tissues could be indicative of the initiation of abnormal cell differentiation. Similarly Nanda et al 27, CK expression was 40% in 10 samples of leukoplakia and 30% in 10 samples of oral submucous fibrosis. Increased expression of CK8 and CK18 was seen in dysplasia, OSF, and OSCC. Staining pattern and intensity showed variations, with intensity of staining in basal and suprabasal layers for CK8 and CK18. Ogden GR 49 observed that simple epithelial keratins (K8, K18, K19) were not confined to the more poorly differentiated tumors. This may be relevant to tumor prognosis.

Sawant S et al 22 analyzed that CK8 and CK18 expression was seen in 50% (31 samples) and 14%(23 samples) samples respectively. In the majority of cases, CK8 and 18 expressions were seen in the suprabasal layers. Fillies T et al3 found detectable levels of CK 8/18 (CK 8/18 $\geq$ 1%) in 54 % (154/287) of the oral SCCs. Kittipong D et al.54 reported that CK 18 expression was 16.7% in the examined 30 samples of lichen planus, suggesting an increased expression in lichen planus.

Makino T et al47 found that positive CK18 expression was identified in 90 (42.9%) cases, and the staining was mainly observed in the cytoplasm of tumor cells. The remaining 120 (57.1%) cases were negative. Furthermore, 85 (40.5%) cases showed positive immunostaining for CK8 in the cytoplasm of tumor cells, whereas 125 (59.5%) were negative. The positive staining for CK18/8 was almost homogeneous at single cancer nest and among different areas (surface, central, and deepest areas) of the cancer lesion. There was a significant correlation between CK18 and CK8 immunostaining ($P < 0.001$); 69 (32.9%) patients showed positive immunostaining for both CK18 and CK8, with largely matching distribution, whereas 104 (49.5%) were negative for both. On the other hand, 37 (17.6%) patients showed discordant immunostaining, including CK18 positive and CK8 negative in 21 cases, and CK18 negative and CK8 positive in 16. Intraepithelial neoplasia (dysplasia) was observed in 55 cases. Among them, CK18 and CK8 expressions were detected in13 (23.6%) and 14 (25.5%) cases, respectively.

Alka D kale et al 23 reported no immunoreactions of CK 8/18 were noted in the oral mucosa of non-neoplastic cases. In OSCC only 10% cases showed immunoreactions whereas in adjacent normal looking mucosa tissues group, 80% of cases were stained positive for CK 8/18.

Mitra S.K. et al35 observed cytokeratin 8 positivity in 34 out of 60 cases (56.67%) of invasive squamous cell carcinomas with maximum intensity in 17/60(28.33%) cases and no expression was seen in control and oral intraepithelial lesions while cytokeratin 18 was expressed in 25 cases (41.67%) out of 60 cases of invasive squamous cell carcinomas with maximum intensity seen in 5/60(8.34%) cases and its expression was also absent in all the benign cases as well as in oral intraepithelial lesions. In well differentiated squamous cell carcinoma, maximum intensity of CK 8 was seen in 8 out of 38(21.05%) cases whereas in poorly differentiated carcinomas, maximum intensity was seen in 3 out of 5 (60%) cases. While reaction intensity of CK18 expression was maximum in 40% cases in poorly differentiated carcinomas as compared to 5.26% in well differentiated squamous cell carcinoma.

Afrem M.C et al36 did not notice any CK18 immunoreactivity in any of the well differentiated tumors. It was present in seven of the eight moderately differentiated cases and in all three poorly differentiated cases of lingual squamous cell carcinoma. Thus, the CK18 immunoreactivity was correlated with the clinical stage, the degree of differentiation and the invasion pattern. The more advanced the clinical stage, greater the number of immunoreactive cells. Meanwhile, high scores of CK18 reactivity were obtained in poorly differentiated forms compared to the well-differentiated ones and in the invasion patterns of higher degree compared to those of lower degree (the significant difference being between the 1st and 4th degrees).

In the present study, out of 10 we found expression of CK8 and CK18 in the 2 normal mucosa samples mainly in basal and

suprabasal layers. Matthias C et al found that no expression of CK8/18 was seen in control tissue, except for very mild and incidental expression in cells of stratum basale. Alka D et al in 201223 also studied the 10-normal mucosa sample adjacent to SCC and found that the expression of CK8 and CK18 in the complete epithelium, expression of CK 8/18 was enhanced in the epithelium of majority of tissues of ANM (80%). Its enhanced expression was also noted in all ANM showing severe dysplasia. Previous research also supports that expression of CK 8/18 is enhanced in leukoplakia with dysplasia than compared to without dysplasia and seems to play an important role in progressing to OSCC. Similarly Makino T et al47 found that non-cancerous squamous epithelium showed no immunohistochemical for both CK18 and CK8, but adjacent proper oesophageal glands always showed strong immunostaining for both, which served as an internal positive control.

Gires et al 56 said that CK8 was not detected in normal mucosa except for a very mild and incidental expression in cells of the stratum basale but high levels in carcinoma cells. Sharda S et al22 found that CK18 expression was seen in 5 cases (50%) of normal, 6 cases (60%) of OSCC and six cases (12%) of OSF. The staining was seen only in the basal layer of OSF and basal and suprabasal layers of normal and OSCC. CK8 staining was not seen in any of the normal tissues studied while 5 (50%) of OSCC and 5 (10%) of OSF exhibited CK8 staining. No staining was seen in normal while basal and suprabasal staining was seen in OSF and OSCC.

Sawef et al 50 found Keratins 8/7 (identified by CAM 5.2) were expressed in the basal cells of approximately a third of the normal biopsies from oral cancer patients. Morgan et al detected keratins CK8/K7 are not expressed by normal oral keratinocytes although occasional staining of Merkel cells in the basal region has been observed.

Frohwitter G et al19 showed that low-molecular weight CK8/18 and 19 cytokeratins, whose expression is a hallmark of glandular tissues, are not physiologically expressed in normal squamous epithelium, but may be expressed during carcinogenesis. In our study, CK8 and CK18 expression was increased with increased dysplasia (Mild<Moderate<Severe).and from severe dysplasia to Oral squamous cell carcinoma (moderately differentiated<well differentiated). Grading of color intensity of IHC also increased (No Color<Yellow<Light Brown<Dark Brown) with increasing dysplasia.

Mohanta A et al57 studied that CK8/18 serve as markers for simple epithelial differentiation. Moderated and poorly differentiated OSCC may express CK8 and sometimes CK7 and CK18. It is also reported that CK8 is associated with transformation and increased malignant potential of oral squamous epithelial cells.

Matthias K et al53 studied oral leukoplakia (n = 19) and some cases were stained with CK8 specific antibodies. In normal or hyperplastic leukoplakia (n = 9) 7 tissue specimens were negative for CK8 (78%) and 2 specimens expressed CK8 weakly in 10% and to intermediate levels in 5% of the cells, respectively. Four out of six (66.7%) dysplastic leukoplakia expressed CK8 to intermediate (++) or even strong (+++) levels in varying percentages of cells. Four leukoplakia samples actually represented carcinomas of small sizes (T1-2) and all expressed CK8 to strong (+++) levels. Six lymph node metastases (LNM) from primary carcinomas of different origin expressed CK8 to intermediate or strong levels and with a percentage range of positive cells from 5–100%. Interestingly, three out of six LNM displayed a strong (+++) K8 expression in 100% of cells.

CK18 and its complementary partner CK8 are members of the intermediate-filament expressed in simple or single-layer epithelial tissues of the body. CK18 has been reported to regulate intracellular signaling and apoptosis thereby influencing tumor growth and invasive capabilities. Conflicting results are observed among invitro and in vivo studies and the precise role of CK18 is yet to be deciphered. We have observed that the mean score of CK8/18 expression has progressively increased from normal mucosa to potentially malignant disorders to oral squamous cell carcinoma which is in contrast to other reported studies by Makino T48, Matthias C54, Nanda K.S, Alka D et al 23 Frohwitter G et al19.

We observed that the expression of CK8/18 was altered in OPMDs and OSCC. It was consistently expressed among our study samples and did not lose expression with severity of dysplasia or differentiation of OSCC. Lower sample size with unequal sample distribution might be our limitation along with the usage of only CK8/18 markers for this study. The samples of our study group were also obtained from a restricted geographical zone of Sriganganagar, Rajasthan, Western India.

Most of the reported studies show that prognosis is poorer with increased CK8/18 expression and hence, we are planning to follow-up our study group for the progression or relapse and correlate it with the immunohistochemical expression. We are also planning to do a multi-centric study involving regional cancer institute's and other dental colleges in our vicinity to add valuable data in this field.

Conclusion

CK8/18 are site-specific and dependent on differentiation. Strong expression was noted in OPMD and OSCC in our study

group. There was a progressive increase in its expression from the normal mucosa to OPMD to OSCC but our values did not reach statistical significance.

Hence, we suggest that CK8/18 could be used as a marker for the visualization, diagnosis and assessment of prognosis for potentially malignant lesions and oral cancers. Evaluation of CK8/18 in adjacent normal mucosa, recurrent lesions and post-operative/post-therapy lesions can enhance our knowledge on tumorigenesis.

Further investigations will be necessary to explore the mechanisms of CK8/18 expression as well as their exact functions in such cancerous lesions to improve our understanding on tumor biology and its behaviour. This will give an important clue for therapeutic intervention, treatment monitoring and to improve patient survival. Newer treatment strategies could be formulated based on the modification of CK8/18 in OSCC.

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Conflicts of interest

There are no conflicts of interest

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