

Association of Clinical Stages of Oral Submucous Fibrosis to Cytokeratin 19 Immunohistochemical Staining

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ABSTRACT

Introduction: Oral submucous fibrosis (OSF) is the most common potentially malignant disorders which is widely spread and has high malignant transformation rates. The use of immunohistochemical (IHC) studies has become one of the stepping stones to establish new surrogate markers to analyze the malignant transformation of a potentially malignant disorder to malignancy.

Oral submucous fibrosis has been classified as stages 1, 2, and 3 according to severity of fibrosis and hyalinization, but the intensity of IHC staining of cytokeratin 19 (CK19) among these stages has not been done to analyze and establish the fact whether the intensity increases according to clinical stages.

Materials and methods: Thirty-five cases of OSF were taken according to the clinical stages and an IHC study was done using CK19. The p-value was calculated using Fisher's exact tests.

Results: Among 35 cases, 6 cases of stage 1 showed 33.3% of negative, 0% of mild, 50% of moderate, and 16.6% of intense staining. Twelve cases of stage 2 showed 16.6% of negative, 25% of mild, 33.3% of moderate, and 25% of intense staining. Seventeen cases of stage 3 showed 5.8% negative, 47% mild, 35.2% moderate, and 11.7% intense staining. The overall and comparative p-values were insignificant.

Conclusion: Since the overall and comparative p-values were insignificant, the present study shows that there is no relation between the clinical stages of OSF and the CK19 staining. Hence, there is no increase in intensity according to clinical stages.

Clinical significance: Establishing a direct relation between clinical stages of OSF and many cellular and extracellular proteins through IHC studies would make a well-established proof directly correlating the severity and staging in mouth opening to intensity of CK19. In this study, p-values were less and hence the study showed an insignificant clinical outcome.

Keywords: Cytokeratin 19, Immunohistochemistry, Normal oral mucosa, Oral squamous cell carcinoma, Oral submucous fibrosis.

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INTRODUCTION

Oral potentially malignant disorder (OPMD) is a common term suggested to replace oral precancer, including both oral precancerous lesions and oral precancerous conditions. The OSF is one of the OPMDs.¹

Cytokeratins are one of the groups of intermediate filament (IF) proteins spread in the cytoplasm of eukaryotic cells that help to maintain the cytoskeletal framework of these cells and are more specifically expressed in epithelial tissues.²

Cytokeratins forming the cytoskeletons of the epithelial cells come under several molecular types. Their patterns of expression vary depending upon the type of epithelial cells and therefore, they may be used as potential markers of cell differentiation and malignant transformation. Cytokeratin 19, which is a type I (acidic) keratin, is considered as the smallest keratin and is unique in context to lack in the carboxyterminal, non- α -helical tail domain, which is more typical for all other keratins.³

Recently, various IHC markers are showing great promise in helping to predict prognosis and response therapy, even at a very early point of tumor development or it may be a premalignant condition like OSF.⁴ Alteration of CK19 expression has been shown in leukoplakia and oral cancer.⁵ Alteration in the intensity of IHC staining of CK19 among clinical stages of OSF has not been done to analyze and establish the fact whether the intensity increases according to clinical stages. This study is carried out to characterize the CK19 profile in OSF based on the clinical stages of OSF and establish the fact as to whether this could be used as a surrogate marker for malignant transformation, and if the results hold positive, then CK19 can be the ideal surrogate marker that can be used to analyze and characterize the rate and conversion of OSF to oral squamous cell carcinoma (OSCC) or any malignant lesion, correlating it with clinical stages of OSF.

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MATERIALS AND METHODS

Hypotheses to be Tested

Whether CK19 can be used as a substitute marker for malignant transformation of OSF based on whether the intensity increases according to clinical stages.

Source of Data

Total of 35 cases of OSF were taken for the study. Confirmed OSF cases were taken for this study and were collected from the Oral Pathology Department archives, Yenepoya Dental College, Mangaluru, Karnataka, India, Century Dental College, India, and some private pathology labs in Mangaluru, India. These histologically confirmed cases were divided according to clinical stages according to the reference from their previous recorded clinical history and biopsy forms.

Inclusion and Exclusion

Thirty-five cases of histologically confirmed cases of OSF were included and sections of size less than 2 µm were excluded.

Collection of Data

Sections of 3 µm thickness were first prepared from the formalin-fixed, paraffin-embedded tissue blocks, then sections were mounted on poly L-lysine-coated slides for CK19 expression, and IHC staining was done using polymerase technique. The primary and secondary antibody for the study was obtained from Biogenex company, Bengaluru, India.

All the sections were coded before staining for CK19. Evaluation of CK19 was done under a light microscope under 10× objective and the intensity of staining of epithelium was assessed as (–) negative, (+) mild, (++) moderate, and (+++) intense, the sections were decoded, and results were tabulated.

The intensity of staining was analyzed by the percentage of tissue section stained per slide.

- If no tissue is stained—Negative
- If 1/3 of the epithelium tissue is stained (approximately 33%)—Mild
- If 2/3 of the epithelium tissue is stained (approximately 66%)—Moderate
- If more than 2/3 of the epithelium tissue is stained (above 66%)—Intense.⁴

Two independent observers were made to evaluate these slides and when discrepancy existed, a third pathologist was made to evaluate the slide to arrive at the consensus conclusion; 35 cases of OSF section were analyzed after dividing them based on clinical stages

as stages 1, 2, and 3 respectively. A total of 35 sections altogether were analyzed.

Statistical Analysis

The results were calculated using Fisher’s exact test. All statistical analyses were done using STATA 14. If the p-value was less than 0.05, then it was considered statistically significant.

Photomicrographs were obtained using CX-(Olympus) microscope.

RESULTS

Out of 35 cases of OSF, 6 cases were stage 1, 12 cases were stage 2, and 17 cases were stage 3. Six cases of stage 1 showed 33.3% of negative, 0% of mild, 50% of moderate, and 16.6% of intense staining. Twelve cases of stage 2 showed 16.6% of negative, 25% of mild, 33.3% of moderate, and 25% of intense staining. Seventeen cases of stage 3 showed 5.8% negative, 47.6% mild, 35.2% moderate, and 11.7% intense staining. All data are supported by Table 1 and Figures 1 to 3.

After Fisher’s exact test was done, the p-value was 0.309, nonsignificant. Since the total p-value was 0.309 and

- Stage 1 vs stage 2 0.692

Table 1: CK19 staining in different clinical stages of OSF

Lesions/ control	CK19 expression				Total
	Negative	Mild	Moderate	Intense	
OSF stage 1	2	0	3	1	6 (100%)
OSF stage 2	2	3	4	3	12 (100%)
OSF stage 3	1	8	6	2	17 (100%)
Total	5	11	13	6	35 (100%)
p-value = 0.309					

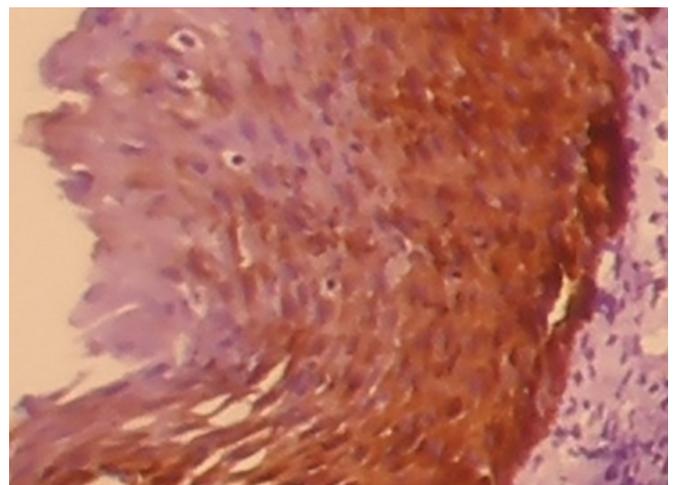


Fig. 1: Mild staining in OSF tissue

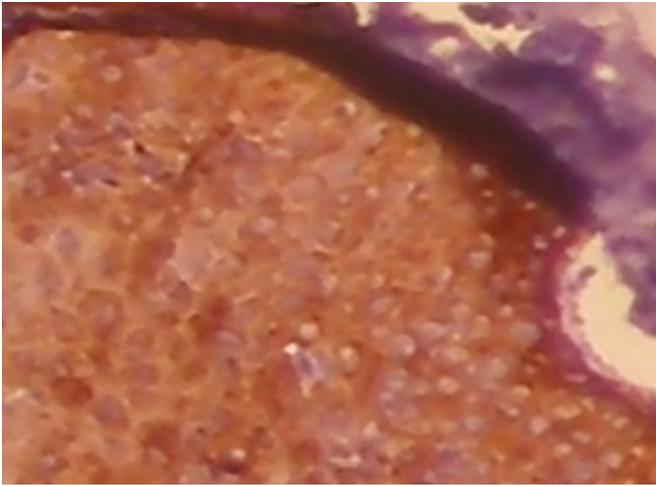


Fig. 2: Moderate staining in OSF tissue

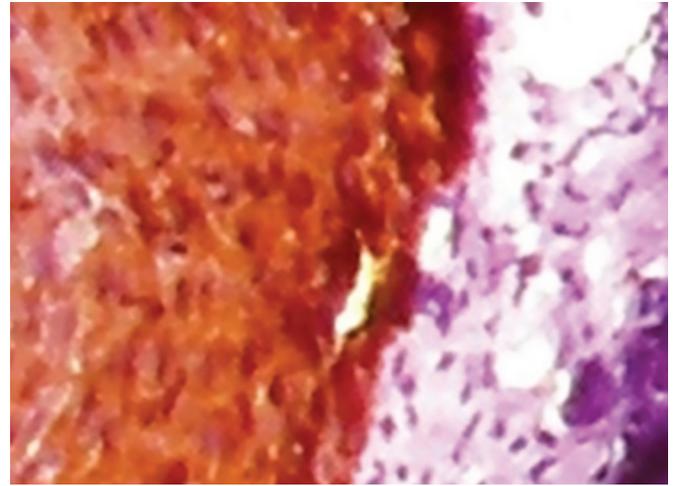


Fig. 3: Intense staining in OSF tissue

- Stage 1 vs stage 3 0.512
- Stage 2 vs stage 3 0.945

there was no recorded significant difference between the intensity levels and was considered statistically insignificant.

DISCUSSION

Chewing tobacco or Gutka has become very popular among locals in India, and betel chewers often use Gutka as well. Recently, Gutka has been officially banned from the Indian market, but chewing tobacco containing betel quid has become a continued popular habit in South Asia. Betel is now widely available in the Western world also.⁶

Over the last 40 years, many theories linking OSF to various risk factors have been proposed. Spicy, pungent foods and irritants, such as supari (areca nut), paan (betel leaves), tobacco (through chewing or smoking) have all been incriminated as causative agents. Systemic factors, such as nutritional deficiency, genetic predisposition, and autoimmunity have also been proposed in the pathogenesis of OSF. However, the exact etiology of OSF is still not known, and no conclusive evidence has been found despite many extensive investigations on implicated factors.⁷

The common early symptoms and signs would be burning sensation, blanching of oral mucosa, oral ulceration, and xerostomia. The oral mucosa in the later stages would become stiff and opaque due to the formation of fibrous bands on the buccal mucosa, soft palate, lips, and tongue, causing restrictions in mouth opening, difficulty in mastication, speech, and swallowing. Further development of fibrous bands in the lip makes the lip heavy, tough, and hard to move. Depapillation of the tongue at the tip and lateral margins may take place with fibrosis of the ventral mucosal part. The soft palate may become widely blanched, and the uvula may shrink in 17% of

cases, making them appear as a small bud or hockey stick.⁸

Clinically, OSF cases can be divided into three clinical stages according to their ability to open the mouth, as follows.

1. Stage I: Mouth opening ≥ 45 mm
2. Stage II: Restricted mouth opening 20 to 44 mm
3. Stage III: Mouth opening ≤ 20 mm⁹

In our study, the clinical staging of OSF was done according to the above-described classification.

The OSF shows typical histopathological features having an atrophic epithelium with juxtaepithelial hyalinization and collagen of different density.⁹

The OSF could be considered as the disease of collagen metabolic disorder. The increased collagen production and decreased collagen degradation will cause an increased collagen deposition in the oral soft tissue that leads to fibrosis.

The OSF tissues in our study showed the above histologically confirmed characteristics with varying levels of fibrosis and hyalinization.

Leukoplakia is a precancerous lesion which is found in more than 25% of people having OSF. This lesion can cause speech and hearing problems due to the involvement of the tongue and the eustachian tubes.¹⁰

In view of increasing numbers of patients of OSCC who have associated OSF in the clinical practice, the incidence of OSCC concomitant with OSF seems to be much higher than that reported in the literature.¹¹

A study done by Prakash et al¹² to determine the p53 expression by IHC and evaluate their potential as surrogate biomarkers of malignant transformation in OSF showed that 73% cases of OSF were positive for p53 marker and thus p53 marker can be used as a diagnostic and prognostic marker for early and prompt treatment planning.

Vimentin, a class 2 IF, is mainly expressed in cells of mesenchymal origin. It is also involved in cell growth, cell cycling, and tumor differentiation. In a study done by Nayak et al¹³ comparing the expression of vimentin in different histological grades of OSF, the staining intensity of vimentin remarkably increased statistically in OSF cases when compared with normal control. A marked increase in the staining intensity of vimentin was also seen in the fibroblasts of severe cases of OSF.

In our study, staining of CK19 proteins was to be analyzed. It is a member of the keratin family. The keratins are IF proteins, which is the reason for the structural integrity of epithelial cells and have been subdivided into CKs and hair keratins. Keratin 19 comes under type I keratin.

In a study done by Babiker et al,¹⁴ expressions of p16 and CK19 markers were different in the same tumor. CK19 positivity was related to age and p16 showed negligible expression. The expression of p16 significantly decreased, while CK19 increased with the different tumor grades. Findings also showed a progressive loss of p16 and significant expression of CK19 according to tumor metastasis.

In an IHC study by Ranganathan et al,⁵ remarkable difference in the CK staining was seen in between normal mucosal tissue, OSF, and OSCC. The OSF showed an increased intensity of staining for panCK and high molecular weight CK, aberrant expression of CK8 and low expression of CKs 5 and 14.

Lalli et al,¹⁵ in their IHC study, observed a high expression of KI and K10 staining in the suprabasal layer, induction of K6 in the basal layer, and complete absence of K19 in the epithelium. In a group of most severe OSF cases (14%), K17 expression was completely absent in the basal layer, defining them to be at most risk to undergo malignant transformation. There was no expression of K8, K18, K7, and K9 and the expression of K4, K13, K14, K15, and K16 did not change in OSF.

In an IHC study by Tilakaratne et al,¹⁶ the data indicated that hypoxia-inducible factor (HIF-1 α) is increased at both protein and messenger ribonucleic acid levels in OSF and the association with dysplasia is statistically significant. The HIF-1 α could be playing a role in malignant transformation of OSF. Further increased expression of HIF-1 α could be also contributing to the progression of fibrosis. It could be quite possible to use HIF-1 α as a marker for malignant transformation of OSF considering the results.

Unlike the above studies,^{6,14-16} our study did not show a significant difference in CK19 staining pattern overall as well as when compared with each stage. More studies comparing clinical stages of OSF and staining of as many cellular and extracellular proteins to establish a direct relation are required.

CONCLUSION AND CLINICAL SIGNIFICANCE

Establishing a direct relation between clinical stages of OSF and many cellular and extracellular proteins through IHC studies would make a well-established proof directly correlating the severity and stages of mouth opening to intensity of CK19, which tends to increase toward metastasis and malignancy as shown in previous studies.¹⁵

Hence, there is a huge scope for further studies into other CKs, intracellular and extracellular proteins on different clinical stages of OSF for exploring and establishing markers which can be used for diagnostic, prognostic, and excellent treatment planning.

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