Original Article

# Expression of p63 and Proliferating Cell Nuclear Antigen in Oral Submucous Fibrosis

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ABSTRACT

**Aim:** Oral squamous cell carcinoma (OSCC) may be preceded by potentially malignant disorders such as oral submucous fibrosis (OSMF). p63 can detect genetic changes in OSMF and it facilitates early detection of malignant transformation, whereas proliferating cell nuclear antigen (PCNA) is a marker of proliferation and may prove to be a useful objective indicator of the biological behavior of various tumors. The aim of this study was to investigate the expression and pattern of the intensity of p63 protein and PCNA in normal oral mucosa and OSMF using immunohistochemistry (IHC) and to correlate the expression of these biomarkers. **Materials and Methods:** A total of 15 archival paraffin-embedded blocks obtained from our department, which were histopathologically diagnosed early OSMF ($n = 4$), intermediate OSMF (n = 4), and advanced OSMF ($n = 2$) and normal mucosa ($n = 5$), were taken as the standard for comparison. p63 and PCNA positivity was analyzed using Kruskall-Wallis test followed by pairwise comparison using Mann-Whitney U test. The pattern of staining and intensity was compared using Chi-Square test for which Statistical Package for the Social Sciences (SPSS, v 22.0, IBM Corporation, Armonk, New York) was used. **Results:** All samples showed positive staining for p63 and PCNA. A statistically significant difference ($P < 0.05$) was seen between the frequency of occurrence of p63 and the PCNA pattern of expression among all the groups. The intensity of staining was mild to intense in the basal layer, as there was a progression toward the severity of the disease. Almost 75.4% correlation existed between p63 and PCNA, with high correlation and marked relationship. **Conclusions:** The OSMF is considered a potentially malignant disorder that has the potential to get transformed into OSCC. The malignant transformation is often associated with changes at the genetic level, and these are reflected by the altered expression of proteins. Our results showed that biomarkers such as p63 and PCNA are significant in predicting the malignant transformation in OSMF, so in future they may serve as a prognostic tool in the early detection of malignancies.

**Keywords:** *OSMF, p63, PCNA, potentially malignant disorders*

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## Introduction

The OSCC is the most common malignant neoplasm of the oral cavity,[1] and it is usually preceded by potentially malignant disorders.[1-3] In spite of the various kinds of research related to the diagnostic modalities and management aspects of OSCC, the

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mortality rates are still low with a five-year survival rate and these are directly related to the stage at which the initial diagnosis is made.[4,5] "Oral potentially malignant disorders" (OPMDs) was defined by the WHO in 2005 as "the risk of malignancy being present in a lesion or condition either at the time of initial diagnosis or at a future date."[6]

OSMF is a potentially malignant disorder that is predominantly seen in people of Asian descent.[7] It is a chronic debilitating disease of the oral cavity that is associated with arecanut (betel-nut) chewing, affecting all parts of the oral mucosa and oro-nasopharynx.[5] Areca nut is the main etiological factor for OSMF. Increased collagen synthesis or reduced collagen degradation is the possible mechanism in the development of the disease.

The condition is well recognized for subsequent malignant transformation and is, therefore, considered a potentially malignant disorder; it has a malignant transformation rate to OSCC of about 7–30%.[8]It has been stated that atrophic changes in the mucosa predispose to malignant changes in the epithelium of OSMF, as atrophy may result from cytotoxicity by the components in areca quid.[9] The malignant potential in OSMF was first described by Paymaster in 1956 and emphasized subsequently by other authors based on clinical and epidemiological grounds.[3,10] Progression to cancerous cells from the normal cells is attributed to the accumulation of genetic alterations. These alterations can be in the form of mutations in a few genes (tumor suppressor genes or oncogenes, gain or loss of chromosomal material, or loss of heterogygosity).

Various molecular biomarkers have been proposed to be of value in the diagnostic and prognostic evaluation of OPMDs, as they may undergo malignant transformation.[2,9,11] Therefore, the development of markers that can detect an early genetic change in these lesions would be of great value.[11]Among the pool of various biomarkers, one of them is p63, having a remarkable structural similarity to p53,[12] which is known to be expressed in embryonic tissue and the basal regenerative layers of epithelial tissue in the adult and is also expressed in various benign and malignant lesions of the body, including lesions of the oral cavity.[11] It is suggested that p63 in concert with p53 also regulates cell proliferation and differentiation and may have a role in potentially malignant and malignant lesions of the oral cavity.[13]

The human p63 gene located on chromosome 3q28 encodes N-terminal transactivation domain (TAP63 with amino acids 1–59), a core DNA binding domain (amino acids 142–321), and a carboxy-oligomerization domain (amino acids 353–397).[2] P63 is important for the development of limb, tongue, teeth, hair, mammary and prostate glands, and sweat and lacrimal glands. In postnatal epidermis, p63 expression is restricted to nuclei of basal cells of normal epithelia (skin, oral mucosa, esophagus, tonsil, prostate, urothelium, ectocervix, and vagina) and to certain populations of basal cells in glandular structures of prostate, breast, and bronchi.[11]

Immunohistochemical methods for detecting proliferation markers, such as PCNA, have been widely used to identify the genetic abnormalities that accompany premalignant and malignant progression, and they can be used with both paraffin-embedded and frozen tissues.[9]

The PCNA is the auxiliary protein of DNA polymerise-5 that is concentrated in the nucleus. It plays an important role in DNA synthesis, DNA repair, cell cycle progression, and cell proliferation. The PCNA levels are very low in inactive cells, but they are produced before DNA replication. The PCNA is said to be a more sensitive index of proliferating marker, as this protein increases at the G1 and S phase and decreases at the G2 phase of the cell cycle. An increase in PCNA expression has been observed as the tissues progressed from the normal epithelium to hyperplasia and dysplasia, potentially malignant and malignant lesions of the oral cavity.[10]

The purpose of this study was to investigate the expression and pattern of the intensity of p63 protein and PCNA antigen in normal oral mucosa and OSMF using immunohistochemistry and to clarify the correlation of the expression of these cell cycle regulatory proteins.

## Materials and Methods

### Sample collection and preparation

A total number of 15 archival paraffin-embedded blocks obtained from the Department of Oral Pathology and Microbiology were studied. The sections were stained with H & E, which consisted of histopathologically diagnosed cases of early OSMF ($n$ = 4), intermediate OSMF ($n$ = 4), and advanced OSMF ($n$ = 2) based on the histological classification by Utsunomiya *et al.*[14] Normal mucosa ($n$ = 5) was taken as the standard for comparison. Confirmed cases were stained with markers p63 and PCNA (BioGenex polymer – horseradish peroxidase [HRP] IHC detection system) for IHC analysis.

Antigen retrieval was done using the EZ-Retriever system technique. Assessment of antigen-expressing cells was performed using a light microscope (Multi-viewing Microscope Model CXR5) at ×400 magnification.

### Immunohistochemical procedure

1. The tissue sections were cut to 3–4 µm thickness and were taken on the coated slides, and the slides were marked.
2. Baking was done at 60°C for 1 h, after which it was dipped and deparaffinized in xylene for 10 min (two changes)
3. Slides were rehydrated through graded alcohols (three changes) and running tap water.
4. The slides were not allowed to dry at any point.
5. Antigen retrieval was performed by placing the slides in AR1 for 10 min at 90°C and in AR2 for 15 min at 98°C in the EZ-Retriever System.
6. Slides were cooled to room temperature.
7. After washing the slides with phosphate buffer solution, peroxide block was applied for 10 min.
8. Hydrophobic barrier was drawn around tissue using a PAP pen to avoid the spreading of reagents away from the tissue.
9. Power block was applied for 10 min, and primary antibody (monoclonal mouse anti-PCNAantibody [MIB-1] for PCNA and antihuman p63 protein [DO-7; DAKO] for p63) was added and incubated for 1 h.
10. The slides were washed in buffer for 3 min (3 times), and super enhancer (secondary antibody) was applied for 20 min.
11. Again, the slides were washed in buffer for 3 min (3 times), and poly HRP enzyme polymer reagent was applied for 30 min and washed with buffer.
12. Diacetylbromoacetic acid was added to the slide for 5 min, and the slides were washed in running tap water.
13. The slides were dipped in Mayer's hematoxylin for less than a minute and washed under running tap water.
14. Finally, the slides were dehydrated in ascending grades of alcohol and then to xylene and mounted with diphenylene phthalate xylene.

### Interpretation of the p63 and PCNA

The slides for p63 and PCNA were observed under a light microscope with a magnification of ×400. The tissue samples were thoroughly examined, and the pattern of expression was analyzed semiquantitatively by counting the number of positive cells per 100 basal, parabasal, and spinous cells and it was recorded as a percentage.

### Evaluation of staining

The intensity of staining was graded based on the subjective evaluation of color exhibited (brown color) by antigen, antibody, and chromogen complex as mild (+, light brown color), moderate (++, dark brown color), or intense (+++, very dark brown color). The distribution of staining was graded as confined only to basal layer, parabasal layer, and spinous layer of the epithelium. Only nuclear staining of epithelial cells was observed, and the nuclei with clear brown color, regardless of staining intensity, were considered as positive. Known positive immunostaining slides were used as positive controls.

The parameters used to analyze the expression of both p63 protein and PCNA antigen were as follows:

1. Pattern or distribution of expression in the epithelial layers
2. Intensity of staining in each slide
3. The percentage of positive cells or labeling index (LI).

### Statistical procedures

Data obtained were compiled on a Microsoft Office Professional Plus 2010, Version 14.0.7128.5000: for windows, One Microsoft Way Redmond, WA 98052 and were subjected to statistical analysis using Statistical Package for the Social Sciences (SPSS v 22.0, IBM). Comparison of differences in means of % of positive p63 cells between all the groups such as control/normal, early, intermediate, and advanced was done using Kruskall-Wallis test, followed by pairwise comparison using Mann-Whitney U test. Also, the comparison of differences in means of % of positive PCNA cells between all the groups such as control/normal, early, intermediate, and advanced was done using Kruskall–Wallis test.

The pattern of staining of p63 and PCNA and intensity was compared with all the groups using Chi-square test. The correlation between the percentage of positive cells (labeling index) of p63 and PCNA was also calculated, where r value and R2 value was calculated. Using a regression model, an estimate of relationship of p63, PCNA is also depicted. For all the statistical tests, $P < 0.05$ was considered statistically significant, keeping α error at 5% and β error at 20%, thus giving a power to the study as 80%.

## Results

The Kruskal–Wallis one-way ANOVA test showed that there was a significant difference of mean LI ($P = 0.020$*) between the different groups (normal, early OSMF, intermediate OSMF, and advanced OSMF) [Table 1]. The distribution of cases according to groups and the pattern of staining for p63 antibody showed a statistically significant difference between the frequency of occurrence of the p63 pattern of expression among all the groups overall ($P = 0.002$) [Table 2]. Groups

**Table 1: Comparison of four groups with respect to labeling index by Kruskal–Wallis one-way ANOVA test**

| Variables | Groups | *N* | Mean | Std. deviation | Kruskall–Wallis test value | *P* value |
|---|---|---|---|---|---|---|
| p63 | Normal | 5 | 1.946000 | .5634536 | | |
| | Early OSMF | 4 | 26.170000 | 15.7975990 | | |
| | Intermediate OSMF | 4 | 33.990000 | 8.1057058 | | |
| | Advanced OSMF | 2 | 37.470000 | 10.4510382 | | |
| | Total | 15 | 21.687333 | 17.2975636 | 9.813 | 0.020* |
| PCNA | Normal | 5 | 8.180000 | 6.1418238 | | |
| | Early OSMF | 4 | 20.765000 | 9.6549314 | | |
| | Intermediate OSMF | 4 | 21.177500 | 8.6550655 | | |
| | Advanced OSMF | 2 | 29.205000 | 5.6922096 | | |
| | Total | 15 | 17.805333 | 10.3210512 | 7.672 | 0.053# |

PCNA = proliferating cell nuclear antigen, OSMF = oral submucous fibrosis

*Statistically significant difference

#Non-significant difference

**Table 2: Distribution of cases according to groups and pattern of staining**

| Variables | Group | B | B + PB | B + PB + SP | Total | Chi-Square value | *P* value |
|---|---|---|---|---|---|---|---|
| p63 | Normal | 5 | 0 | 0 | 5 | | |
| | Early OSMF | 1 | 3 | 0 | 4 | | |
| | Intermediate OSMF | 0 | 1 | 3 | 4 | | |
| | Advanced OSMF | 0 | 0 | 2 | 2 | | |
| | Total | 6 | 4 | 5 | 15 | 20.250 | 0.002* |
| PCNA | Normal | 5 | 0 | 0 | 5 | | |
| | Early OSMF | 3 | 1 | 0 | 4 | | |
| | Intermediate OSMF | 0 | 3 | 1 | 4 | | |
| | Advanced OSMF | 0 | 1 | 1 | 2 | | |
| | Total | 8 | 5 | 2 | 15 | 13.219 | 0.040* |

PCNA = proliferating cell nuclear antigen, OSMF = oral submucous fibrosis

*Statistical significant difference ($P < 0.05$)

**Table 3: Groups versus intensity basal layer**

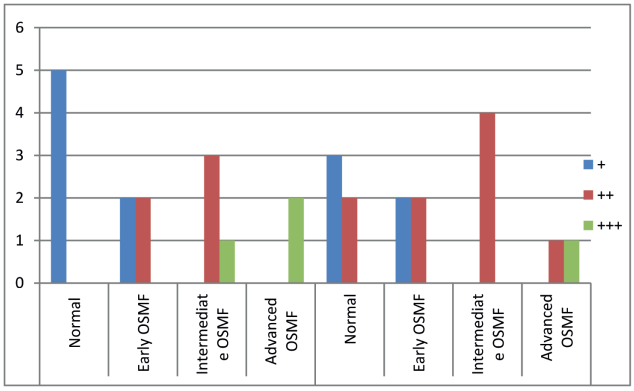
| Variables | Group | + | ++ | +++ | Total | Chi-Square value | *P* value |
|---|---|---|---|---|---|---|---|
| p63 | Normal | 5 | 0 | 0 | 5 | | |
| | Early OSMF | 2 | 2 | 0 | 4 | | |
| | Intermediate OSMF | 0 | 3 | 1 | 4 | | |
| | Advanced OSMF | 0 | 0 | 2 | 2 | | |
| | Total | 7 | 5 | 3 | 15 | 18.857 | 0.004* |
| PCNA | Normal | 3 | 2 | 0 | 5 | | |
| | Early OSMF | 2 | 2 | 0 | 4 | | |
| | Intermediate OSMF | 0 | 4 | 0 | 4 | | |
| | Advanced OSMF | 0 | 1 | 1 | 2 | | |
| | Total | 5 | 9 | 1 | 15 | 13.031 | 0.043* |

PCNA = proliferating cell nuclear antigen, OSMF = oral submucous fibrosis

*Statistical significant difference ($P < 0.05$)

versus p63 intensity basal layer showed a statistically significant difference ($P = 0.004$) between the frequency of occurrence of the p63 pattern of expression in the basal layer among the groups overall [Table 3, Graph 1].

A pairwise comparison of four groups with respect to the LI of p63 by Mann–Whitney U-test showed a statistically significant difference in LI when pairwise comparisons were done between control and early OSMF and also between control and intermediate OSMF ($P < 0.05$); however, no difference was seen with other pairs ($P > 0.05$) [Table 4]. The distribution of cases according to groups and LI of p63 showed a statistically significant difference ($P = 0.007$) between the frequency of occurrence of the p63 pattern of expression coded as per three groups (6%–25%, 26%–60%, and 61%–99%) [Table 5, Graph 2]. A comparison of four groups with respect to LI of PCNA by Kruskal–Wallis one-way ANOVA test showed the nonstatistical significant




**Graph 1:** p63 and PCNA intensity basal layer

**Table 4: Pairwise comparison of four groups with respect to labeling index by Mann–Whitney *U* test**

| Group | Mean % | *U* value | *Z* value | *P* value |
|---|---|---|---|---|
| Normal | 1.946 | 0.000 | −2.449 | 0.016* |
| Early | 26.17 | | | |
| Normal | 1.946 | 0.000 | −2.449 | 0.016* |
| Intermediate | 33.99 | | | |
| Normal | 1.946 | 0.000 | −1.936 | 0.095# |
| Advanced | 37.47 | | | |
| Early | 26.17 | 6.000 | −0.577 | 0.686# |
| Intermediate | 33.99 | | | |
| Early | 26.17 | 2.000 | −0.926 | 0.533# |
| Advanced | 37.47 | | | |
| Intermediate | 33.99 | 3.000 | −0.463 | 0.800# |
| Advanced | 37.47 | | | |

OSMF = oral submucous fibrosis

*Significant difference ($P < 0.05$). Statistical significant difference was seen when normal mucosa group was compared with early and intermediate OSMF groups

#Non-significant difference ($P > 0.05$). Statistical non-significant difference was seen when normal mucosa group was compared with advanced OSMF group; early OSMF group when compared to intermediate and advanced OSMF group and when intermediate OSMF group was compared to advanced OSMF group

difference ($P = 0.0532$) between different groups [Table 1]. The distribution of cases according to groups and the pattern of staining for PCNA showed a statistically significant difference ($P = 0.040$) between the frequency of occurrence of the PCNA pattern of expression among all the groups [Table 2].

Groups versus the PCNA intensity basal layer showed a statistically significant difference ($P = 0.043$) between the frequency of occurrence of the PCNA pattern of expression in basal layer among the groups overall [Table 3, Graph 1]. The distribution of cases according to the groups and LI of PCNA showed a statistically significant difference ($P = 0.005$*) between the frequency of occurrence of the PCNA pattern of expression coded as per three groups (0%–5%, 6%–25%, and 26%–60%) [Table 5, Graph 2]. Correlation between p63 and PCNA in predicting the disease is 0.754 (75.4%). Table 6 shows regression estimation, that is, a way to show relationship when one increases what happens to the other variable. Here, we have got a positive relationship, that is, positive correlation with *r* value as 0.754, $P = 0.001$*($P < 0.05$). *Statistically significant difference suggesting high correlation and marked relationship [Tables 6 and 7].

## Discussion

OSMF is considered to be PMDs with the potential to get transformed into OSCC. The malignant transformation is often associated with changes at the genetic level that are reflected by the altered expression of proteins.[15] Hence, molecular biological markers have been suggested to detect the abnormal cell proliferation and the early genetic changes in PMDs, which may have the potential to progress to malignancy.[11]

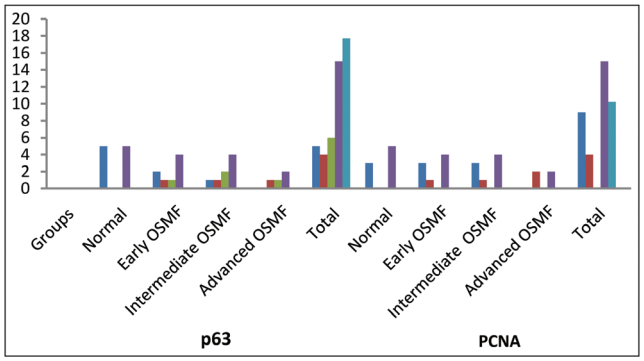
p63 is secreted in the embryonic ectoderm from the seventh to the eleventh day.[2,11] p63 has two transcriptional sites that generate transcripts encoding proteins with or without an *N*-terminal TA. Proteins

**Table 5: Distribution of cases according to groups and labeling index**

| Variables | Groups | 0–5% | 6–25% | 26–60% | 61–99% | Total | Chi-Square value | *P* value |
|---|---|---|---|---|---|---|---|---|
| P63 | Normal | 0 | 5 | 0 | 0 | 5 | | |
| | Early OSMF | 0 | 2 | 1 | 1 | 4 | | |
| | Intermediate OSMF | 0 | 1 | 1 | 2 | 4 | | |
| | Advanced OSMF | 0 | 0 | 1 | 1 | 2 | | |
| | Total | 0 | 5 | 4 | 6 | 15 | 17.708 | 0.007* |
| PCNA | Normal | 2 | 3 | 0 | 0 | 5 | | |
| | Early OSMF | 0 | 3 | 1 | 0 | 4 | | |
| | Intermediate OSMF | 0 | 3 | 1 | 0 | 4 | | |
| | Advanced OSMF | 0 | 0 | 2 | 0 | 2 | | |
| | Total | 2 | 9 | 4 | 0 | 15 | 10.219 | 0.005* |

PCNA = proliferating cell nuclear antigen

*Statistically significant difference seen between the frequency of occurrence of p63 and PCNA pattern of expression coded as per groups (0–5%, 6–25%, 26–60%, and 61–99%) among all the groups overall ($P < 0.05$)




**Graph 2:** Distribution of cases according to the groups and labeling index of p63 and PCNA

**Table 6: Correlation between percentage of positive cells (labeling index) of p63 and PCNA**

| Variable | Percentage of positive cells for PCNA | | |
|---|---|---|---|
| | Spearman rank correlation | *r* value | *P*-level |
| Percentage of positive cells for p63 | | 0.754 | 0.001* |

PCNA = proliferating cell nuclear antigen

*Statistically significant difference ($P<0.05$). 75.4% correlation exists between p63 and PCNA that suggested high correlation with marked relationship

**Table 7: Dependent variable PCNA versus independent variable p63**

| Dependent variable: PCNA% of positive cells | | | | | | |
|---|---|---|---|---|---|---|
| Model summary | | | | | | |
| *R* square | *F* | df1 | df2 | Sig. | Constant | *b*1 |
| .561 | 16.628 | 1 | 13 | .001 | 8.111 | .447 |

The independent variable is p63% of positive cells

Positive correlation *R* square value 0.561, *r* value as 0.754, *P* value as 0.001*

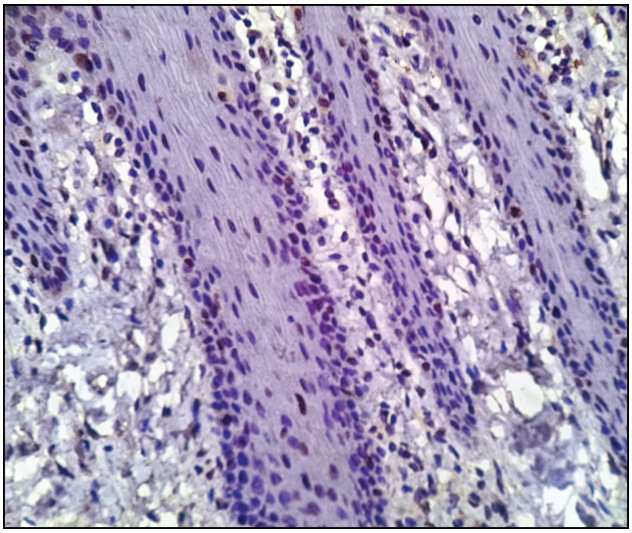


**Figure 1:** Photomicrograph showing p63 expression in normal oral mucosa seen in basal layer of the epithelium (IHC Stain, X400)

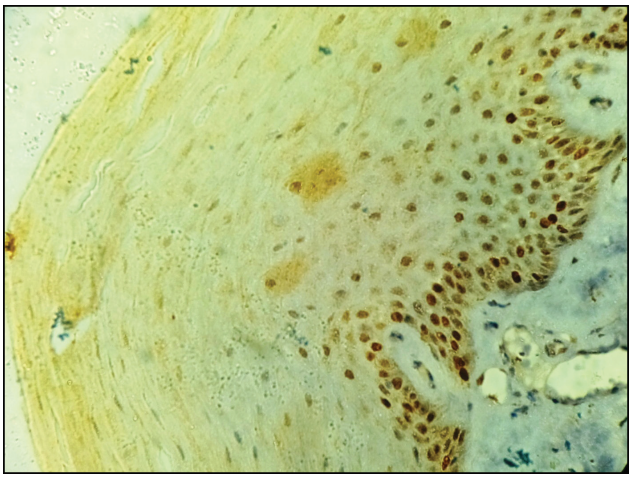


**Figure 2:** Photomicrograph showing p63 expression in early OSMF seen in basal and parabasal layer of the epithelium (IHC Stain, X400)

with TA are termed TAP63, and proteins without it are termed ΔNP63. TAP63 isoforms transactivate P53 downstream targets, induce apoptosis, and mediate cell cycle control. However, ΔNP63 isoforms have functions opposite to TAP63 by acting as oncoproteins. In normal oral mucosa (NOM) and reactive epithelial hyperplasia, ΔNP63 expression has been reported in the basal layer, decreasing toward the middle-third of the epithelium that is also seen in our study where we found expression in the basal layer of the epithelium [Figure 1]. However, in the dysplastic lesions, expression has been observed up to the spinous layer or sometimes the entire thickness of the epithelium that is seen in our study also. The primary function of p63 is to regulate epithelial stratification by maintaining the proliferative undifferentiated state of basal keratinocytes, thereby establishing the basal status of the epithelium. Therefore, p63 is postulated as the first gene product that differentiates between stem cells and transient amplifying cells in the stratified squamous epithelium.[2,11]

In our study, p63 positivity was seen in all cases of OSMF [Figures 2 and 3], which was in accordance with Sharda *et al.*[2] and Sinha *et al.*[11] p63 expression was seen in the basal layer of the normal epithelium, in agreement with other studies carried out by Sinha *et al.*,[11] Sharda *et al.*,[2] and Haniffa *et al.*[12] The pattern among the groups showed a gradual increase in the number of cells with positive nuclei from the basal to the spinous layer. We found that the pattern of expression of p63 was higher as the grade of OSMF progressed from early to advanced as compared with the normal oral epithelium, which was in agreement

with Bavle *et al.*, where they found that the expression of p63 was higher as the grade of OSMF progressed from early to advanced.[16]

Comparison of p63 staining intensity revealed that p63 expression in the basal layer was weak (+) in normal/control cases compared with early, intermediate, and advanced OSMF, which showed moderate (++)-to-intense (+++) staining intensity. The report just cited and the present study suggested that molecular changes predisposing toward malignant transformation can also take place in the OSMF epithelium, even in the absence of morphologically apparent dysplastic changes.

Cellular proliferation is the important indicator to detect the biological aggressiveness of a malignant lesion. The dysregulated proliferation may be a significant change to determine the potential prognosis of various malignant tumors.[17] The PCNA is a nuclear protein and marker of cell proliferation.[9] It is well known now that areca quid contains cytotoxic components, along with compounds such as betel nut, areca nut, catechu, influorescence of piper betel, and lime that can stimulate cell proliferation. Thus, the increase in PCNA expression in OSMF might be induced by direct stimulation from mitogen-like compounds present in areca quid or by regenerative proliferation after cell death.[9] Increased PCNA expression has also been seen as tissues progressed from the normal epithelium to malignancy.[10,18]

In the normal epithelium, PCNA expression was confined to the basal cell layer with no positivity in the superficial layer.[14,19] In the present study also, all samples of normal mucosa showed positive staining with PCNA in the basal cell layer of the epithelium [Figure 4]. This might be due to physiological proliferative activity in the basal cell layer. PCNA positivity was seen in all cases of OSMF [Figures 5 and 6]. Chi-square test showed a statistically significant difference (P value-0.040) between the frequency of occurrence of the PCNA pattern of expression among all groups. We found that as we moved toward the severity of stages of OSMF the positive expression of PCNA moved toward the spinous layer of the epithelium, which was in agreement with Mandeep *et al.*, where they found PCNA staining in a spinous layer in a few cases of OSMF.[9]

A comparison of PCNA staining intensity revealed that PCNA expression in the basal layer was weak (+) in control cases compared with the OSMF that showed moderate (++) to intense (+++) staining intensity. Thus, by this we can conclude that the intensity of expression in the basal layer is directly proportional to

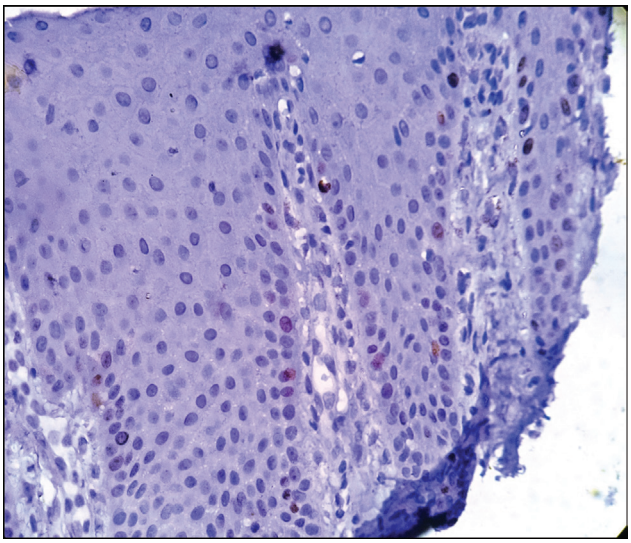


**Figure 4:** Photomicrograph showing PCNA expression in normal oral mucosa seen in basal layer of the epithelium (IHC Stain, X400)

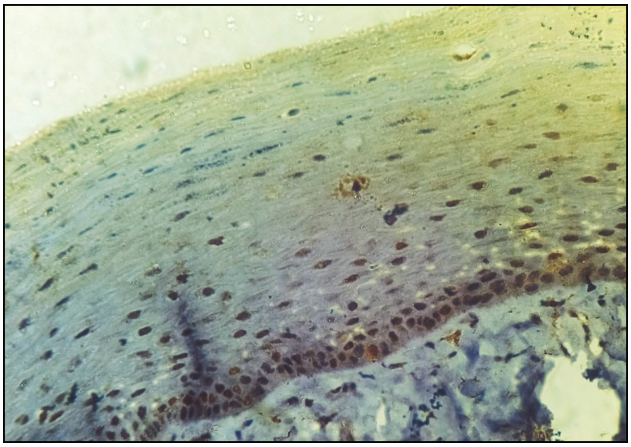


**Figure 3:** Photomicrograph showing p63 expression in advanced OSMF seen in basal, parabasal, and spinous layers of the epithelium (IHC Stain, X400)

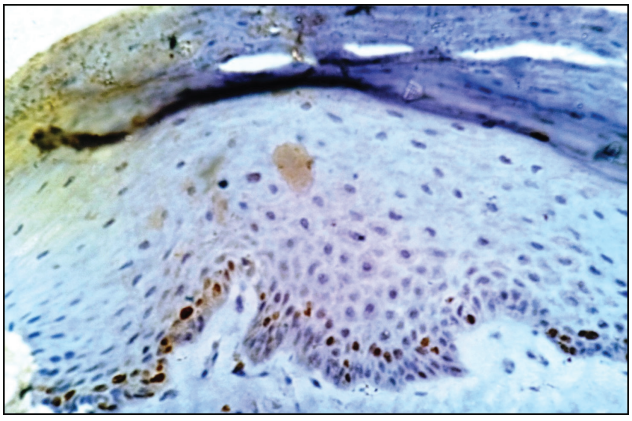


**Figure 5:** Photomicrograph showing PCNA expression in early OSMF seen in the basal and parabasal layer of the epithelium (IHC Stain, X400)

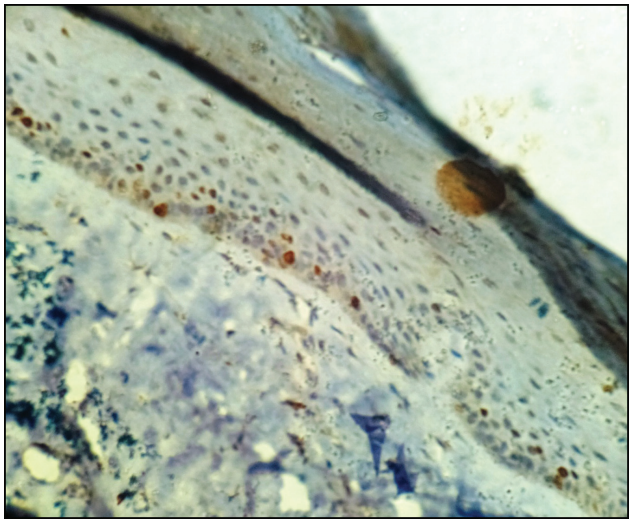


**Figure 6:** Photomicrograph showing PCNA expression in advanced OSMF seen in basal, parabasal, and spinous layers of the epithelium (IHC Stain, X400)

the severity of the condition. Similar observations have been seen by Roopavathi *et al.,*[10] where they found that the intensity of staining in basal cells of OSMF varied from moderate to high and the percentage of cells exhibiting high intensity staining was directly related to the grade of OSMF and the basal layer in OSMF had significant proliferative activity.[10]

In our study, percentage positivity of PCNA increased with the advancement of stage of the disease; compared with normal oral mucosa, similar observations were also seen in the study conducted by Baveja *et al.*[20]

In the present study, Spearman rank co-relationship showed 75.4% correlation between p63 and PCNA. The regression curve estimation has shown even a positive correlation with $R2 = 0.561$, $R = 0.754$, and $P = 0.001$*, which is a high correlation and marked relationship. Thus, we can suggest that alterations in p63 may lead to increased cellular proliferation in OSMF.

Overall, our observations contribute to findings that p63 and PCNA immunoexpression could be used as a specific biomarker for lesions that are at high risk of malignant transformation. These biomarkers can be used as prognostic markers of proliferation and might be used as a supplement of histopathological assessment in the prognosis of potentially malignant disorders such as OSMF.

### Limitations and strengths

The sample size used in this study was small, which was our biggest limitation. However it can provide a base for future researchers using a larger sample size to obtain more reliable data.

## Conclusion

From our study, we hypothesized that a significant increase in the expression and intensity of p63 and PCNA in OSMF may aid in the early detection of PMDs at risk of developing OSCC. Therefore, patients showing the overexpression in pattern and intensity of these biological markers are at a considerably high risk of malignant transformation and should be closely monitored. Our study also showed a correlation of 75.4% between p63 and PCNA, suggesting that altered p63 gene product promotes uncontrolled cellular proliferation that is considered the most important biological mechanism of carcinogenesis and a change in the expression of its related protein is the most important index for detecting the potential of malignant changes in OSMF.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

### Authors' contributions

All authors contributed in the study and design of the manuscript.

### Ethical policy and institutional review board statement

Approved by the Institutional Ethical Committee.

### Patient declaration of consent

All the patient consents were taken according to the protocol.

### Data availability statement

Available and stored with the first and corresponding author.

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