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Original Article

Immunohistochemical Evaluation of P63 in Clinical and Histopathological Stages of Oral Leukoplakia: A Tertiary Care Hospital Study

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Abstract

Introduction: Leukoplakia is the precancerous lesion par excellence of the oral mucosa. The risk of malignant transformation of leukoplakia with dysplasia has been reported as high as 43%. Leukoplakia shows histologic findings such as epithelial hyperplasia, hyperkeratosis, and hyperparakeratosis, with or without epithelial dysplasia or carcinoma. Epithelial development and subsequent formation of squamous epithelial tissues in humans are a complex biological process. Stratified epithelia (the epidermis) require p63 expression for its development. P63 initiates epithelial stratification during development and maintains the proliferative potential of basal keratinocytes in the mature epidermis, thereby playing a dual role. P63 isoforms lacking the N-terminal transactivating domain, such as ΔNp63, induce a functional block against p53 and TAp63/p73 activities, and its overexpression is observed in many squamous cell carcinomas. These data suggest the oncogenic properties of p63 and its potential to increase cell proliferation and antagonize apoptosis. Due to the infrequent mutation of p63 and its overexpression in various cancers, considerable interest has recently been focused on p63.

Purpose: The purpose of this study was to evaluate the correlation of p63 expression with histological state and clinical type of oral leukoplakia.

Materials and Methods: Patients reporting to the outpatient department of the tertiary care hospital over 1 year were examined clinically. The cases showing oral leukoplakia lesions were considered for this study. Thus, the study comprised 58 points clinically showing oral leukoplakia. Thirty-eight of these were selected for quantification of p63 positive cells.

Results: P63 expression, according to the WHO grading system, indicated an increase in expression pattern from no to mild to severe dysplasia. Multiple overall comparisons were made using one word: ANOVA. The test results were highly significant, with a value (95% confidence level) equal to 0.000. P63 expression was evaluated using the N-Par Mann–Whitney U-tests between groups.

Keywords: Cancer, Dysplasia, Leukoplakia, P63, Precancer, Verrucous

INTRODUCTION

The terms pre-cancer, precursor lesions, premalignant, intra-epithelial neoplasia, and potentially malignant have been used in literature to broadly describe clinical presentations that may potentially become cancer.[1] Leukoplakia is the most common premalignant, potentially malignant, or precancerous lesion of the oral mucosa.[2] Leukoplakia is a clinical term for characterizing a white lesion with an increased risk for malignant potential.[3] Warnakulasuriya *et al.*, in 2007, defined leukoplakia as "*recognizable white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer.*"[4] Most authorities regard leukoplakia as a dynamic rather than a static lesion, but this is usually in terms of its progression and development of malignancy.[5] There is a wide range in the malignant transformation rate of leukoplakia. According to various studies, 16–62% of oral carcinomas are associated with

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leukoplakic lesions when diagnosed initially. (Bouquot *et al*., 1988; Gundlach, 1992; Reichart, 1998; and Schepman *et al*., 1999).[6] The rate of malignant transformation varies with location, clinical features, degree of dysplasia, and etiological factors.[7] Seven non-homogeneous leukoplakia has been defined as a predominantly white or white-and-red lesion ("erosive leukoplakia" and "erythroleukoplakia") that may be either irregularly flat nodular ("speckled") or verrucous.[8]

MATERIALS AND METHODS

The study entitled "**Expression of p63 in histological and clinicopathological stages of oral leukoplakia: An immunohistochemical study**" was conducted in the Department of Oral Pathology and Microbiology at the Institute Hospital.

Patients and Experimental Design

Patients coming to the outpatient department of the Institute Hospital over 1 year from 2011 to 2012 were examined clinically. The cases showing oral leukoplakia lesions were considered for this study.

Inclusion Criteria

Cases clinically showing oral leukoplakia lesions were included in the study. No specific criterion about age, sex, or location of the lesion was applied.

Exclusion Criteria

Population below 10 years of age and pregnant females were excluded from the study. Any form of chemotherapy, radiotherapy, systemic ailments, and the presence of any other mucosal lesions were excluded from the study. Pregnant patients and ones suffering from leukemia or other hematological disorders, where the biopsy is not indicated and may pose a threat of significant hemorrhage, and patients suffering from acquired immunodeficiency syndrome were excluded from the study. Thus, the study comprised 58 cases clinically showing oral leukoplakia. Thirty-eight of these were selected for quantification of p63 positive cells.

Case History and Clinical Examination

A detailed case history of each patient was recorded, which included chief complaint, age, sex, duration of symptoms, site of the lesion, medical and dental history, and habits. The study's purpose and procedure were explained to the patient. Only those patients who agreed to participate in the study were chosen. Informed consent was obtained from them. These cases were evaluated by detailed extraoral intraoral examination to arrive at the working or provisional diagnosis.

Clinical Staging of Leukoplakia

A clinical assessment of leukoplakia was done on the criteria provided by Axell *et al*. It was divided into two homogenous groups (Low-risk) and non-homogenous (High-risk) types. Homogenous leukoplakia consists of two types – (1) mild or thin leukoplakia and (2) thick leukoplakia. Non-homogenous leukoplakia consists of three types – (1) granular or nodular, (2) verrucous, and (3) speckled, which was graded by LSCP classification and staging system by Van der Waal *et al*. (1997) [Table 1].

Biopsy Procedure

Population with clinically significant leukoplakia lesions were biopsied. All the cases were subjected to routine hemograms before the biopsy procedure. Intraoral periapical or occlusal radiographs or orthopantomograms were advised before excision, wherever necessary.

A sterile gauze pack was placed between the affected mucosa and the teeth in the wound region. Patients were given post-operative instructions, prescribed antibiotics, analgesics, and anti-inflammatory medications and recalled after 1 week.

Microscopic Examination of the Lesions

A detailed histopathological examination of each lesion under a light microscope was done. Based on microscopic structure, the lesions were classified as hyperkeratotic with no dysplasia or mild dysplasia. Moderate dysplasia, severe dysplasia, and carcinoma *in situ*.

Histopathological Grading of Leukoplakia

Histopathologic assessment of lesions in patients clinically diagnosed as leukoplakia was done for the degree of epithelial dysplasia according to the WHO 2005 classification scheme. Based on dysplastic features (architectural + cytological disturbance), epithelial dysplasia is usually divided into

Table 1: LSCP classification of Leukoplakia

L	S	C	P
L=Extent of the lesion	S=Site of lesion	C=Clinical aspect	P=Stages of dysplasia
L0=No lesion	S1=All oral sites, except for the floor of the mouth and tongue	C1=Homogenous	P1=No dysplasia
L1=Lesion <2 cm	S2=Floor of the mouth and tongue	Cz=Non homogenous	P2=Mild dysplasia
L2=Lesion 2–4 cm	Sx=Not specified	Cx=Not specified	P3=Moderate dysplasia
L3=Lesion >4 cm			P4=Severe dysplasia
Lx=Not specified			Carcinoma *in situ*

L=extent of leion, S=site of lesion, C=clinical aspect of lesion, P=stages of dysplasia

five categories. No dysplasia (squamous hyperplasia): The architecture shows regular stratification without cellular atypia. Mild: Dysplastic features limited to the lower third of the epithelium. Moderate: Dysplastic features extending into the middle third. Severe: Dysplasia greater than two-thirds of the epithelium. Carcinoma *in situ*: Full or almost full-thickness architectural abnormalities in the viable cellular layers accompanied by pronounced cytologic atypia with atypical mitotic figures and abnormal superficial mitoses. The histopathologic features were numbered as per Van der Waal criteria.

Staging of Leukoplakia

Staging is only performed in leukoplakias that have been examined histopathologically [Table 2].

Immunohistochemistry Procedure

To evaluate the distribution and density of p63-positive cells in oral leukoplakia specimens, 38 specimens were subjected to immunohistochemistry using the p63 antibody. The procedure for the same is as follows:

1. 3-micron sections were cut from paraffin blocks and mounted on super frost slides
2. Immunohistochemical study was carried out using the polymer labeling technique (dako envision)
3. Sections were dewaxed and washed in alcohol. Antigen retrieval was performed in a decloaking chamber (Biocare) with ten mM Citra solution at 125°C temperature for 30 s, followed by 900°C for 10 s.
4. Slides were cooled naturally and brought to room temperature.
5. Slides were placed inside the Dako Autostainer Universal Staining System (Automated *et al*.)
6. Endogenous peroxidase was blocked using 0.3% hydrogen peroxide in methanol at room temperature for 10 min.
7. Slides were washed briefly with phosphate-buffered saline (PBS) and incubated with primary antibody (p63) for 60 min.
8. Sections were washed with PBS and incubated with the polymer for 30 min.
9. Sections were washed with PBS. Diaminobenzidine was used as the chromogen in hydrogen peroxide for 10 min.
10. Finally, the sections were counterstained with Mayer's hematoxylin and mounted in Distyrene, Plasticiser, and Xylene (DPX).

For the quantitative analysis of p63 cells, immunostained slides were examined under high power (magnification: ×400) of a light microscope (Olympus CH 20*i*). When the slides were evaluated, nuclear immunoreactivity was considered, and two methods scored p63 expression:

1) P63 positive cells were counted manually in ten randomly selected high-power fields. The slide was moved in a single direction (from right to left) to avoid repetition of already examined fields. Number p63-cells were counted as a mean number of p63 positive cells/high power field.
2) Considering the intensity of nuclear staining (0–3), the thickness (from 0 [one-third of the epithelial layer] to 3 [all the thickness of the epithelial layer]), and the gradient of p63 expression in the epithelial layer (from 0 [no expression] or 1 [conserved gradient of expression] to 3 [complete loss of the gradient of expression]). The score was defined as the sum of these three criteria (0–9).

Control Tissues for Immunohistochemistry

Both positive and negative controls were set. Breast tissue was used as a positive control. With patients' consent, standard mucosa samples, which would otherwise be discarded, were obtained from five patients undergoing tooth extraction to serve as negative controls. The control specimens were subjected to routine fixation and processing, followed by hematoxylin and eosin staining. Finally, they were brought to the same immunohistochemistry procedure.

RESULTS AND OBSERVATIONS

P63 positive cells were counted in basal, suprabasal, and total layer ans of the epithelium for various clinical types of leukoplakias. The expression patterns of basal and total layers significantly increased from homogenous thick to thin to non-homogenous speckled, verrucous, and granular leukoplakias, respectively. One-way ANOVA was used to give an overall comparison between groups. P63 expression in basal, suprabasal, and total showed *P*-value (95% confidence level) of 0.009, 0.000, and 0.001, respectively [Figures 1-8 and Table 3].

P63 expression, according to the WHO grading system, indicated an increase in expression pattern from no to

Table 2: Staging of leukoplakia: Staging is only performed in leukoplakias that have been examined histopathologically

Stage 1	Stage 2	Stage 3	Stage 4
Any L, S1, C~, P1 or P2	Any L, S1, C~, P1 or P2 any L, S2, C1.P1, or P2	Any L, S2, C2, Pl, or P2	Any L, any S, any C, P3, or P4

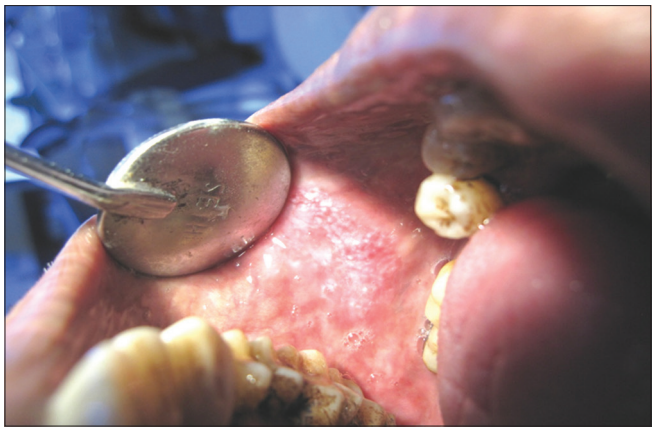

Figure 1: Clinical picture of non-homogenous granular leukoplakia

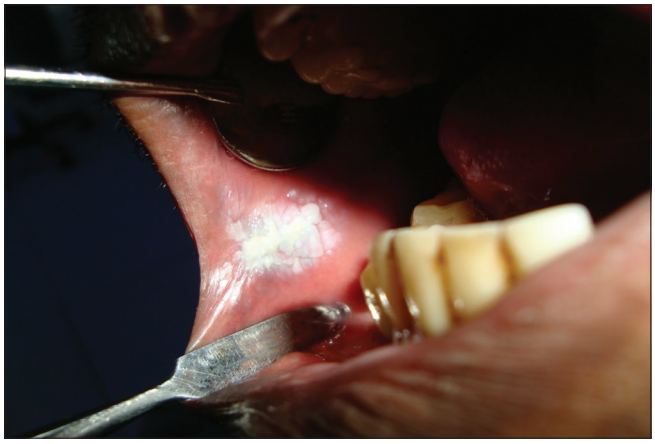

Figure 2: Clinical picture of homogenous thick leukoplakia

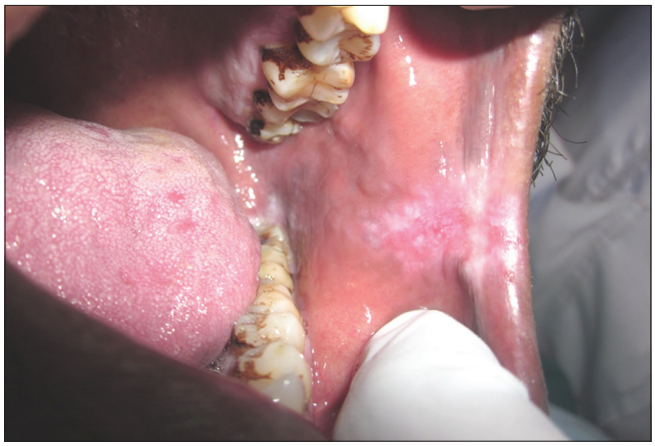

Figure 3: Clinical picture of erythroplakia

mild to severe dysplasia. Multiple overall comparisons were made using one-way ANOVA. The test results were highly significant, with *P*-value (95% confidence level) equal to 0.000. P63 expression was evaluated using the N-Par Mann–Whitney U-tests for between groups [Tables 4-7].

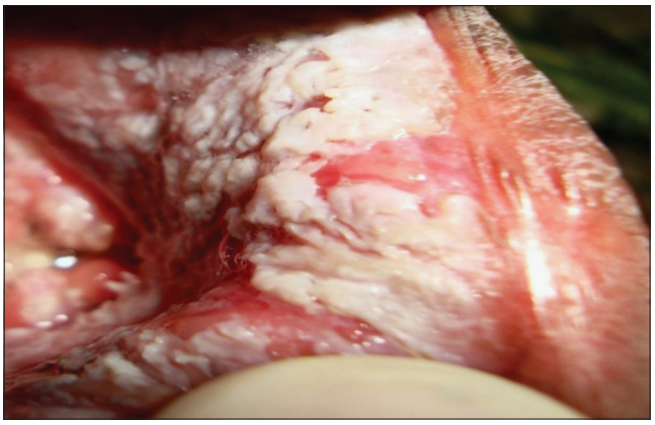

Figure 4: Clinical picture of non-homogenous verrucous leukoplakia

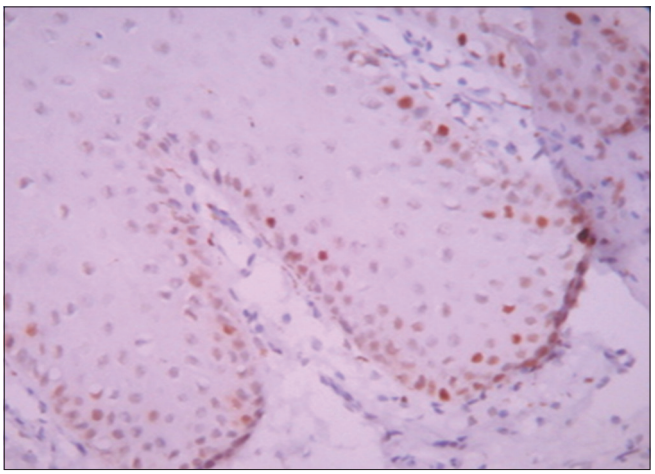

Figure 5: p63 expression in no dysplasia

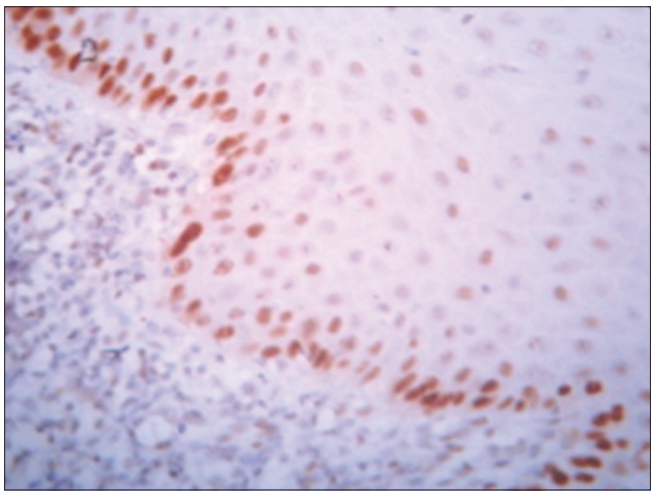

Figure 6: p63 expression in mild dysplasia

DISCUSSION

Oral squamous cell carcinoma (OSCC) has a five-year mortality rate of about 50%.[2] As, As demonstrated by

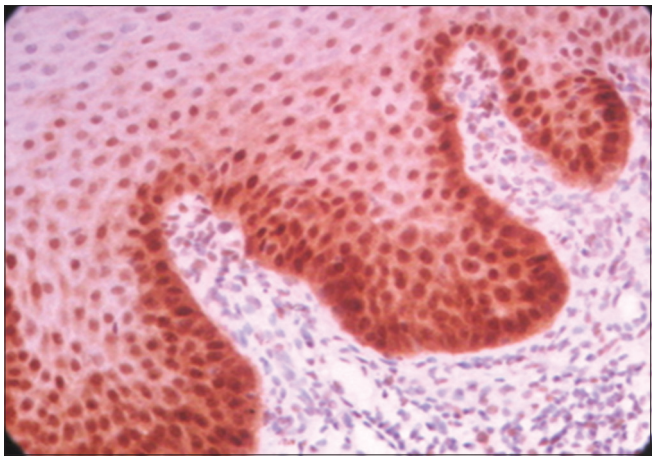

Figure 7: P63 expression in moderate dysplasia

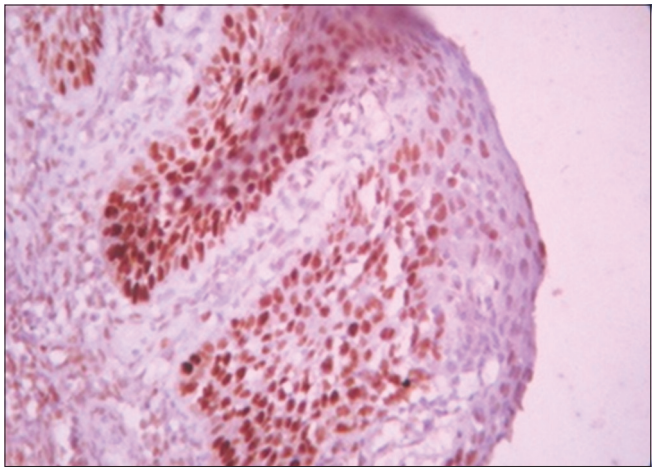

Figure 8: p63 expression in severe dysplasia

Table 3: Distribution of P (histological state) between clinical subtypes of leukoplakia

S. No.	Clinical type	P1	P2	P3	P4
1.	Granular	0.00%	25.00%	0.00%	75.00%
2.	Speckeled	21.10%	63.20%	10.50%	5.30%
3.	Verrucous	100.00%	0.00%	0.00%	0.00%
4.	Thick	65.00%	25.00%	5.00%	5.00%
5.	Thin	58.30%	16.70%	25.00%	0.00%

Table 4: Comparison of P63 basal, suprabasal, and total between clinical subtypes of leukoplakia

S. No.	Clinical Type	P63basal	p63_suprabasal	P63_Total
1.	Granular	166.00	300.75	467.00
2.	Speckled	98.92	128.85	228.00
3.	Verrucous	103.00	92.00	195.00
4.	Thick	85.36	102.00	187.36
5.	Thin	93.50	109.25	202.75

several studies, histopathological grading of leukoplakia has a drawback of interobserver subjectivity. For this reason, it is not easy to establish a strong correlation between the degree of dysplasia and the rate of transformation of leukoplakia. Hence, apart from histopathological findings, the clinical factors associated with leukoplakias, such as site, size and type of leukoplakia, should not be overlooked while predicting the malignant potential of these lesions. Development of the mucosal epithelium requires a series of coordinated events that regulate the proliferation and differentiation of keratinocytes. Recent discoveries have highlighted several genes required for the terminal differentiation of keratinocytes and have explained how the single-layered surface ectoderm commits to initiate stratification during embryogenesis.[9]

A candidate gene involved in this process is the transcription factor p63.[10] p63 is expressed in the surface ectoderm before stratification and continues to be expressed during embryonic development. Expression of p63 is required for the proper formation of limbs, epidermis and other epithelial tissues, including breast and prostate, as evidenced by the phenotypes of p63-null mice and the malformations seen in people that inherit mutations in the p63 gene.[11] Interestingly, it has been shown that there is a preferential expression of DNp63 (compared to TAP) isoforms in squamous cell carcinomas. Thus, overexpression of p63 reflects the immaturity of the tumour cell lineage and may disrupt terminal differentiation and preserve the ability to multiply. So, deregulation of p63 should be an early step in carcinogenesis.[9,13] We have not found any studies correlating the p63 expression pattern with clinical subtypes of leukoplakia. However, various authors have studied the malignant conversion rate of clinical types of leukoplakia. P. C. Gupta *et al.* (1989),[12] who conducted An Epidemiologic Assessment of Cancer Risk in Oral Precancerous Lesions in India with Special Reference to Nodular Leukoplakia, concluded that nodular features should be considered dangerous. Another study conducted by Jose V. Bagan *et al.*[14] found that patients with PVL (Proliferative *et al.*) developed a high frequency of OSCCs, often manifesting several cancers at different oral locations, thus demonstrating the field cancerisation of this entity.

Our results showed a considerable increase in the expression of p63 in all layers of granular leukoplakia compared to other clinical forms. Considering the increased expression of p63 as a prognostic marker of malignant transformation in leukoplakia, it can be summarised that granular leukoplakia has more propensity for malignant conversion. Incidentally, this finding was supported by the fact that out of 4 granular leukoplakia patients we observed, one patient showed conversion of moderate dysplasia to carcinoma in situ in a one-year follow-up. However, this was not part of the study, and patients were not observed.

P63 expression was evaluated between groups using the N-Par Mann-Whitney U tests. A significant difference in the

Table 5: *P*-value (95% confidence level) of p63 expression between various clinical types of leukoplakia

Clinical type of leukoplakia	Homogenous thick	Homogenous thin	Non-homogenous granular	Non-homogenous verrucous	Non-homogenous speckled
Homogenous thick		P63B-0.741 P63S-0.283 P63T-0.215	P63B-0.018 P63S-0.019 P63T-0.013	P63B-0.307 P63S-0.469 P63T-0.310	P63B-0.023 P63S-0.014 P63T-0.010
Homogenous thin	P63B-0.741 P63S-0.283 P63T-0.215		P63B-0.697 P63S-1.000 P63T-1.000	P63B-0.697 P63S-1.000 P63T-1.000	P63B-0.423 P63S-0.275 P63T-0.346
Non-homogenous granular	P63B-0.018 P63S-0.019 P63T-0.013	P63B-0.61 P63S-0.011 P63T-0.017		P63B-0.480 P63S-0.157 P63T-0.157	P63B-0.078 P63S-0.05 P63T-0.07
Non-homogenous verrucous	P63B-0.307 P63S-0.469 P63T-0.310	P63B-0.697 P63S-1.000 P63T-1.000	P63B-0.480 P63S-0.157 P63T-0.157		P63B-0.385 P63S-0.106 P63T-0.264
Non-homogenous speckled	P63B-0.023 P63S-0.014 P63T-0.010	P63B-0.425 P63S-0.277 P63T-0.346	P63B-0.078 P63S-0.05 P63T-0.07	P63B-0.385 P63S-0.106 P63T-0.264	

P63B: p63 basal, P63S: p63 suprabasal, P63T: p63 total

Table 6: Comparison of P63 basal, suprabasal, and total between stages 1, 2, 3, and 4 LSCP grading, given by Vander Wall I; Oral oncology 1997

S. No.	Stages	P63basal	p63_suprabasal	P63_Total
1.	I	69.00	66.25	135.25
2.	II	94.50	117.50	213.00
3.	III	136.33	211.00	347.33
4.	IV	136.90	212.80	348.90

P63 expression pattern was observed between no dysplasia and mild, moderate and severe dysplasias(P<0.5 (95% confidence level)). However, the difference in expression patterns of p63 between mild dysplasia and severe dysplasia and between moderate dysplasia and severe dysplasia was weakly significant. The difference in p63 expression between mild and moderate dysplasia was not significant.

Our findings were similar to those of T. Takeda *et al*. (2006),[15] who found that p63 in the basal layer showed a significant difference between low- and high-grade groups of epithelial dysplasia. The results of Martin Chovanec *et al*. (2005)[16] were also similar, where he found that p63 expression was statistically significant only between Grade 1 and Grade 3.

In the same group of 38 patients, we also studied the clinical parameters of the p63 expression pattern. The non-homogenous leukoplakia (C2) showed a significant increase in expression patterns of p63 in basal, suprabasal and total layers of epithelium compared to homogenous leukoplakia applying Mann Whitney U tests. P63 positive cells were counted in basal, suprabasal and total layers of the epithelium for various clinical types of leukoplakias. The expression patterns of basal and total layers significantly increased from homogenous thick to thin to non-homogenous speckled, verrucous and granular leukoplakias, respectively. These results indicated highly significant differences in expression patterns between various clinical types.

Our findings were similar to those of Kovesi G., Szende B. (2006)*et al*.,[17] who conducted Clinical, histologic, and immunohistochemical studies on oral leukoplakia. The p63 index was 10% homogenous, 5% in nodular or speckled, but nearly 20% in erythroleukoplakia, on average. The results suggested that the characteristic expression of p63 in various forms of leukoplakia may be of prognostic value and that non-homogenous leukoplakia is more aggressive than the homogenous form. On the other hand, contrary to the results of Jose V. Bagan *et al*.[14] the verrucous leukoplakia case we observed showed lesser expression of p63 positive cells compared to other non-homogenous types of leukoplakia, and no statistical.... difference in expression pattern was noted between basal, suprabasal and total layers of epithelium with any other clinical forms of leukoplakia. The variation in this expression pattern compared to our study could be attributed to the small sample size (n=1) of verrucous leukoplakia cases.

When p63 expression was compared in various stages of leukoplakia using LSCP staging as proposed by Van der Waal I,[1] it was seen that p63 expression in basal, suprabasal and total layers of epithelium between Stages I and II, Stage I and III, Stage I and IV, Stage I and III and stage II and IV were statistically significant (P value < 0.05). However, no statistical significance was seen in the p63 expression pattern between all layers of epithelium between stages III and IV. The results indicate that Stage III (oral leukoplakia on tongue and floor of mouth with mild/no dysplasia) has an equal propensity for malignant transformation as Stage IV (lesion anywhere but with moderate, severe

Table 7: Meanwhile, a comparison of P63 basal, suprabasal, and total between various histological grades of leukoplakia according to the WHO (2005) grading system

S. No.	P level	P63basal	p63 suprabasal	P63 Total
1.	No dysplasia	70.46	71.15	141.62
2.	Mild dysplasia	103.93	136.64	241.43
3.	Moderate dysplasia	111.17	153.67	477.00
4.	Severe dysplasia	175.50	301.50	477.00
5.	Carcinoma *in situ*	000	000	000

dysplasia). In our study, we studied histological, clinical, and clinicopathological (LSCP) parameters independently. We found a maximum correlation of p63 expression with LSCP staging.

The results indicate that the malignant transformation of leukoplakia depends on an overall clinicopathological picture of the lesion rather than the presence of epithelial dysplasia alone. The result of the present study infers that surgical management of any leukoplakia lesion on the tongue or floor of the mouth with or without dysplasia should be dealt with with an equal aggressiveness as a stage IV leukoplakia lesion.

CONCLUSION

It has been shown that over-expression of p63 in leukoplakias is correlated to an increased risk of developing oral cancer. The fact that p63 expression is altered in leukoplakia has suggested that it could be an important prognostic marker. The LSCP staging system has been overlooked, and hardly any studies have been conducted to evaluate its role in depicting the malignant potential of leukoplakia cases. To our knowledge, no study has been published comparing the expression of p63 with the clinicopathological stages of leukoplakia. Our results using p63 as a marker have validated the LSCP grading system as a more reliable system for staging leukoplakias compared to the WHO grading system regarding malignant conversion rate. Considering this, we would recommend using LSCP classification universally in our day-to-day oral pathology reporting to provide more relevant data to clinicians. The results of our study, combined with studies involving multiple genetic markers in various precancerous and cancerous conditions, could provide valuable insights into the malignant conversion rate of different clinicopathological stages of leukoplakia in a long-term follow-up.

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