Original Article

Role of p53 and Ki-67 immunomarkers in oral premalignant lesions and squamous cell carcinoma: a hospital based study in BPKIHS

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ABSTRACT

Background: As most of the oral squamous cell carcinoma develop from precursor premalignant lesions, it would be helpful if the malignant transformation is detected early in premalignant state. The objective of the research was to study the role of immunohistochemical expression of p53 and Ki-67 in oral premalignant lesion and squamous cell carcinoma.

Materials and Methods: The expression of immunomarkers p53 and Ki67 were studied on formalin fixed paraffin embedded tissue sections from human oral squamous mucosal lesion for duration of 1 year.

Results: Of total 36 cases, 80% cases of keratosis without dysplasia showed basal pattern of p53 staining while 47.1% cases of squamous cell carcinoma showed p53 staining in all layers of epithelium. The median p53 Labelling Index of squamous cell carcinoma was more than those of keratosis with and without dysplasia though the result was statistically non-significant. 50.0% cases of keratosis without dysplasia and 83.3% cases of keratosis with dysplasia displayed Ki-67 immunostaining confined to basal and suprabasal layer whereas 94.1% cases of squamous cell carcinoma showed Ki-67 positivity in all layers of epithelium. Median Ki-67 Labelling Index increased from keratosis without dysplasia to keratosis with dysplasia to squamous cell carcinoma, difference being statistically significant. A positive and insignificant correlation was observed between p53 and Ki-67 Labelling Index.

Conclusion: Increased expressions of Ki-67 and p53 in oral squamous cell carcinoma compared to premalignant lesion suggest that they may be useful indicator of malignant transformation in dysplastic lesion.

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INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) constitutes the sixth most common cancer worldwide and the third most common cancer in the developing countries. The global annual incidence of oral cancer is about 270,000 cases with 130,000 deaths.[1] The etiology of OSCC is multifactorial and the most important risk factors include personal habits of tobacco use, areca nut chewing and alcohol consumption as well as Human Papilloma virus (HPV) infection, poor oral hygiene and micronutrient deficiencies. Tobacco usage

can cause various genetic and molecular alterations in oral premalignant and malignant lesions.[1,2] Most of the OSCC develops from oral premalignant lesion such as leukoplakia and oral submucous fibrosis (OSMF). The clinical and histopathological changes in these lesions are preceded by changes at molecular level. The identification of these high risk premalignant lesions plays a significant role in reducing the morbidity, mortality and cost of treatment associated with OSCC.[3]

Oral carcinogenesis is a multistep process with accumulation of mutation and epigenetic alteration in oncogenes and tumor suppressor genes leading to disruption of normal cell cycle. These molecular alterations lead to phenotypic change in epithelium from normal to premalignant change to oral carcinoma. Though, there have been various studies in the past to identify molecular markers to predict malignant transformation of premalignant lesions, none of them singly or in combination have been found to be of required sensitivity or specificity to be used in routine practice.[2,4]

TP53, a tumor suppressor gene, regulates cell cycle. Mutation in the TP53 gene is the most common genetic change found in OSCC, which has been seen in 40-50% of the OSCC cases.[5] Though wild type p53 protein has a very short half-life (6-20 min), and is hard to detect in normal tissue, mutation of the TP53 gene result in overexpression and stabilization of the p53 protein, which can be detected by immunohistochemistry.[5]

Ki-67 is a nuclear antigen and is a proliferative marker. Mib-1 is a monoclonal antibody to recombinant parts of Ki-67 antigen, which has been proven equivalent to anti Ki-67, thus facilitating its use in paraffin sections. Ki-67 can be detected immunohistochemically throughout the interphase of the cell cycle, reaching its maximal level during mitosis. Immediately after mitosis, the cellular Ki-67 content decreases due to short half-life (<1 hour) and is not detectable in G0 phase. Moreover in comparison to the counting of mitotic figures, Ki-67 Labelling Index is more sensitive to determine the cell proliferation, as cells in all active phases of cell cycle can be recognized. As the transition of normal oral epithelium to dysplasia to carcinoma is characterized by increased cell proliferation, Ki-67 which is a gold standard proliferative index, has been extensively examined in oral dysplasia and OSCC with increased expression in higher grades of dysplasia.[6-8]

The objective of this study was to study the pattern of staining and Labelling Index (LI) of expression of p53 protein and Ki-67 antigen in oral premalignant lesion and OSCC and to find out the relationship between Labelling Index of p53 protein and Ki-67 antigen in oral premalignant lesion and OSCC.

MATERIALS AND METHODS

A cross sectional study of formalin fixed paraffin embedded tissue sections from human oral squamous mucosal lesion was carried out at Department of Pathology, BP Koirala Institute of Health Sciences (BPKIHS) for duration of 1 year from March 2015 to February 2016. Oral mucosal biopsy specimens of all age groups with oral squamous lesions were received from the Department of Otorhinolaryngology and the Department of Oral and Maxillofacial surgery. Informed consent was taken from all the patients. Oral mucosal biopsy of patients with nonsquamous lesion and patient who refused to give consent was excluded from the study. Ethical Clearance was obtained from Institutional Review Committee, BPKIHS (Code no. IRC/448/015)

For histopathological examination, Hematoxylin and Eosin stained formalin fixed paraffin embedded tissue sections were examined. Known positive immunostaining slides were used as controls. Immunohistochemistry was performed on formalin fixed paraffin embedded tissue sections mounted on APES (3-aminopropyl triethoxy silane) coated slides and were marked for Ki-67 and p53. The slides were left in hot plate for few minutes and then dipped in xylene (2 change, 5 minutes each), rehydrated through acetone (two change, 5 minutes each), alcohol for 5 minutes and running tap water for 5 minutes. The slides were not allowed to dry at any point. Antigen retrieval was performed by placing the slides in citrate buffer (pH 6) in pressure cooker for 15 minutes and was cooled at room temperature. The next steps of staining were then performed in moist chamber. The slides were washed with wash buffer (Tris buffer solution), 4 changes, 5 minutes each, incubated in Hydrogen peroxide block for 30 minutes and again washed with Tris buffer, pH 7.3 (4 changes, 5 minutes each). The primary antibody (monoclonal mouse anti-human Ki67 antigen clone MIB-1) for Ki67 and antihuman p53 (p53 Ab-5, DO-7) for p53 were added and incubated in fridge at 4^0 C overnight. The slides were then washed with Tris buffer and super enhancer (secondary antibody, Ultravision One HRP Polymer) was applied for 30 minutes and washed with Tris buffer (4 changes, 5 minutes each). Di-acetyl bromoacetic acid (DAB) was added to the slide for 10-15 minutes and washed with Tris buffer. The slides were dipped in Harris hematoxylin for 30 seconds and washed in running tap water for 5 minutes, air dried and mounted with DPX.

The prepared slides were examined with Nikon Eclipse E600 microscope under 4x, 10x, 20x and 40x objective lens. Clear brown colour staining of nucleus of epithelial cells was considered positive. All the slides were evaluated by consultant pathologist.

The pattern of staining was graded as Humayun S et al[3]:
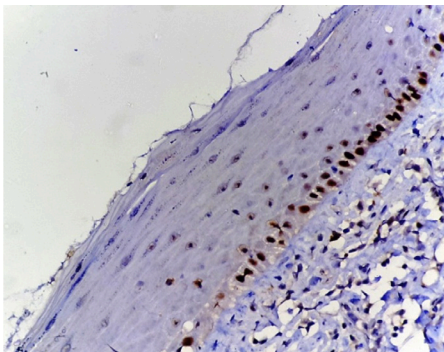
1. Confined to basal layer only,

Figure 1: *Photomicrograph of a case of Keratosis without dysplasia showing positive immunostaining localized at basal layer for p53 (X200)*
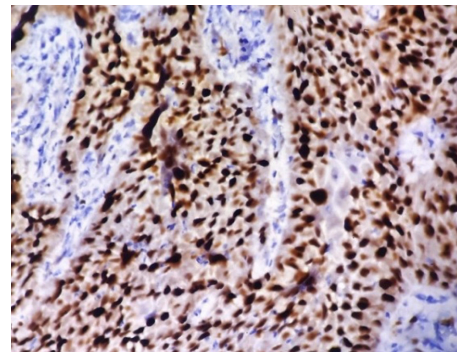

Figure 2: *Photomicrograph of a case of Squamous Cell Carcinoma showing positive immunostaining localized at all layers of epithelium for p53 (X200)*
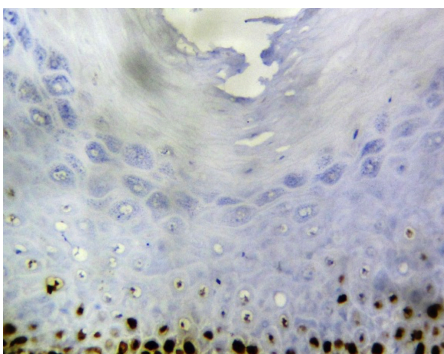

Figure 3: *Photomicrograph of a case of Keratosis without dysplasia showing positive immunostaining localized at basal and suprabasal layer for Ki-67 (X400)*

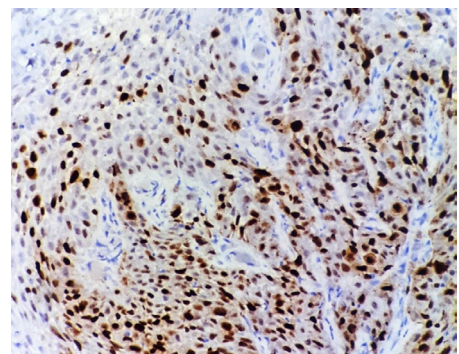**Figure 4:** *Photomicrograph of a case of Squamous Cell Carcinoma showing positive immunostaining localized at all layers of epithelium for Ki-67 (X200)*
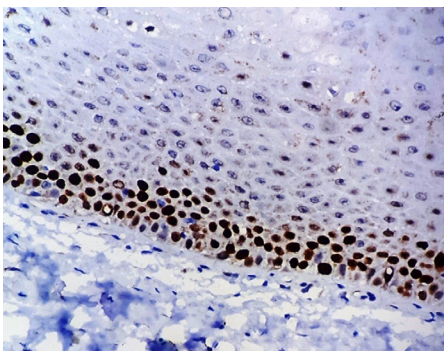

Figure 5: *Case of Keratosis with mild dysplasia showing positive immunostaining localized at all basal and suprabasal layer of epithelium for Ki-67 of epithelium for Ki-67 (X400)*

2. Both basal and suprabasal layer,

3. All layers of epithelium

Labelling index (LI) was calculated by counting the number of positive cells per 1000 squamous cells and was recorded as percentage (Kurokawa H et al[9]).

For statistical analysis, the collected data was entered in Microsoft Office Excel 2007 software. Data analysis was done by using SPSS (Statistical Package for Social sciences) 11.5 version. Chi square test and Kruskal Wallis test were used to find out the significant difference between the two test results at 95% Confidence Interval where $p \leq 0.05$ was considered statistically significant.

RESULTS

A total of 36 cases of oral premalignant lesion and oral squamous cell carcinoma were evaluated for immunohistochemical expression of p53 and Ki-67. The cases were diagnosed as Submucous Fibrosis (1/36), Keratosis without Dysplasia (10/36), Keratosis with Dysplasia (6/36), Oral Squamous Cell Carcinoma (17/36) and Verrucous Carcinoma (2/36). All the cases with histopathological diagnosis of keratosis with dysplasia showed only mild dysplasia.

The study population comprised of 63.9% male and

Table 1: Distribution of cases according to histopathological diagnosis and pattern of staining for p53

Histopathological diagnosis	Pattern of staining for p53				p value
	Negative	Basal	Basal and suprabasal	All layers	
Keratosis without dysplasia	0 (0.0%)	8 (80.0%)	2 (20.0%)	0 (0.0%)	
OSMF	0 (0.0%)	1(100.0%)	0 (0.0%)	0 (0.0%)	
Keratosis with dysplasia	1 (16.7%)	4 (66.7%)	0 (0.0%)	1 (16.7%)	0.080
SCC	4 (23.5%)	3 (17.6%)	2 (11.8%)	8 (47.1%)	
VC	0 (0.0%)	1 (50.0%)	1 (50.0%)	0 (0.0%)	

36.1% female. The most common site involved by the oral premalignant and OSCC was tongue (41.7%), followed by buccal mucosa (36.1%), lip (11.1%) and rest of the site (11.1%).

Most of the cases of keratosis without dysplasia (80.0%) showed p53 positive immunostaining confined to basal layer only (fig.1), 66.7% cases of keratosis with dysplasia showed p53 immunostaining confined to basal layer and 16.7% cases showed p53 immunostaining in all layers of epithelium whereas most of the cases of OSCC included in the study (47.1%) showed p53 positive immunostaining confined to all layers of epithelium (fig.2). None of the cases of Verrucous carcinoma (VC) showed p53 positive immunostaining in all layers of epithelium. (Table 1) The chi-square test did not show any significant association in pattern of staining of p53 between the five groups (p= 0.080). However, significant association in pattern of staining of p53 was found between keratosis without dysplasia, keratosis with dysplasia and SCC (p= 0.020).

The cases with histopathological diagnosis of keratosis without dysplasia showed Ki-67 immunostaining confined to either basal layer (40.0%) or basal and suprabasal layer (50.0%) and none of the cases showed positive staining in all layers of epithelium (fig.3). Moreover, most of the cases (83.3%) of keratosis with dysplasia showed Ki-67 immunostaining located either in basal and suprabasal layers (fig.5) or all layers of epithelium (16.7%). In contrast to keratosis without dysplasia and keratosis with dysplasia, most of the cases of OSCC (94.1%) showed positive immunostaining in all layers of epithelium (fig.4) and only 1 case (5.9%) showed Ki-67 positive immunostaining located in basal and suprabasal layer. (Table 2) The chi-

square test showed significant association in pattern of staining of Ki-67 between the five groups (p<0.001), with extension of Ki-67 from basal to suprabasal to all layers with increase in malignant change. Moreover, a highly significant association was found in pattern of staining of Ki-67 among keratosis without dysplasia, keratosis with dysplasia and SCC (p<0.001).

In the study, median p53 LI of keratosis without dysplasia (20.5%) was greater than that of keratosis with dysplasia (12.0%). The median p53 LI of OSCC (32.0%) was higher than that of keratosis without dysplasia (20.5%) and keratosis with dysplasia (12.0%) and even with 1 case of OSMF (30.0%). p53 LI was more than 60.0% in 35.3% cases of OSCC and less than 25.0% in 35% cases of OSCC. Though median p53 LI was highest ie. 42.5% in VC, the maximum p53 LI was found in OSCC (82.0%). Though the median p53 LI of SCC was higher than that of keratosis without dysplasia and keratosis with dysplasia, there was no statistical difference between the five groups, when all the five groups were compared for median p53 LI by Kruskal Wallis test (p= 0.141). (Table 3)

The median Ki-67 LI was found to be higher with increasing degree of dysplasia, with median Ki-67 LI in keratosis without dysplasia to be only 26.0% in comparison to 42.0% in keratosis with dysplasia. Ki-67 LI was more than 60.0% in 88.2% cases of OSCC. The median Ki-67 LI was highest (75.0%) in SCC among all five groups followed by median Ki-67 LI of VC (55.0%). A significant relationship was found between median Ki-67 LI among the five groups by Kruskal Wallis test (p<0.001) (Table 4) A positive but statistically non-significant correlation was found between p53 LI and Ki-67 LI.

Table 2: Distribution of cases according to histopathological diagnosis and pattern of staining for Ki-67

Histopathological diagnosis	Pattern of staining for p53				p value
	Negative	Basal	Basal and suprabasal	All layers	
Keratosis without dysplasia	1 (10.0%)	4 (40.0%)	5 (50.0%)	0 (0.0%)	
OSMF	0 (0.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)	
Keratosis with dysplasia	0 (0.0%)	0 (0.0%)	5 (83.3%)	1(16.7%)	<0.001
SCC	0 (0.0%)	0 (0.0%)	1 (5.9%)	16 (94.1%)	
VC	0 (0.0%)	0 (0.0%)	2 (100.0%)	0 (0.0%)	

Table 3: Distribution of cases according to histopathological diagnosis and median p53 LI.

Histopathological diagnosis	Median p53 LI (IQR) (Min-Max) (%)	p value
Keratosis without dysplasia	20.5 (9.5-22.25) (6.0-30.0)	
OSMF	30.0*	
Keratosis with dysplasia	12.0 (2.25-25.0) (0.0-28.0)	0.141
SCC	32.0 (6.0-65.5) (0.0-82.0)	
VC	42.5 (42.0-43.0)†	

*As only 1 case of Submucous fibrosis, median cannot be calculated.
†As only 2 cases of VC, only minimum and maximum LI is calculated.

Table 4: Distribution of cases according to histopathological diagnosis and median Ki-67 LI

Histopathological diagnosis	Median Ki-67 LI (IQR) (Min-Max) (%)	p value
Keratosis without dysplasia	26.0 (14.25-34.25) (0.0-48.0)	
OSMF	42.0*	
Keratosis with dysplasia	42.0 (26.75-58.0) (26.0-88.0)	<0.001
SCC	75.0 (69.0-88.0) (21.0-92.0)	
VC	55.0 (45.0-65.0)†	

*As only 1 case of Submucous fibrosis, median cannot be calculated.
†As only 2 cases of VC, only minimum and maximum LI is calculated.

DISCUSSION

The absence of functional p53 leading to genomic instability is a hallmark of human carcinogenesis. Moreover, the basal cell layer is the proliferative component of normal oral epithelium. The presence of proliferating cells beyond the basal layer in both suprabasal and superficial strata of epithelium would indicate abnormal cell proliferation with increasing levels of dysplasia. The mutated forms of p53 and increase in proliferating cells can be detected by immunohistochemical expression of p53 and Ki-67 respectively.[10,11]

In the study, p53 was positive in 76.5% cases of OSCC. A wide geographical variation of p53 expression in oral cancer has been observed in various studies. It is 75% in India, 18% in Srilanka, 27% in Turkey, 44 % in USA, 60% in Netherlands, 63% in Brazil and 70% in Thailand. The variation in the expression of p53 could be the result of differences in genetic predisposition among these populations, oral habits practiced in different geographic regions as well as the variations in methodology applied. However, expression of p53 in 76.5% cases of OSCC in this study is in agreement to that of India, which has similar geography and lifestyle.[12]

The authors Nylander K et al[13] and Cruz I et al[14] have highlighted the importance of suprabasal p53 expression in dysplastic oral lesions and the risk of malignant transformation. The pattern of p53 staining in this study was mostly basal in keratosis without dysplasia (80.0%) and keratosis with dysplasia (66.7%), whereas only 17.6% cases of SCC showed basal staining for p53. 47.1% cases of SCC and 16.7% cases of keratosis with dysplasia showed p53 staining in all layers of epithelium, thus revealing extension of p53 expression from basal to suprabasal layers to all layers of epithelium with propagation of lesion towards carcinoma. These findings were concurrent with the study done by Humayun S et al[3], Kurokawa H et al[9], Patel SM et al[15], Iamaroon A et al.[16]

Though a statistically significant difference between median p53 LI and keratosis without dysplasia, keratosis with dysplasia and OSCC was not observed in the study, the median p53 LI of SCC was more than those of keratosis without dysplasia and keratosis with dysplasia. The p53 LI was more than 60.0% in 35.3% cases of OSCC. These findings are comparable with study by Humayun S et al[3], Santos-Garcia A et al[17], Swaminathan U et al[18], Nasser W et al[19], Iamaroon A et al[16] and Patel S et al.[15] The increase in p53 expression through various grades of OSCC from Well differentiated SCC (WDSCC) to moderately differentiated SCC (MDSCC) to poorly differentiated SCC (PDSCC) has been highlighted by Verma R et al.[20] Motta R et al have shown the importance of p53 overexpression in OSCC and regional lymph node metastasis and consequently worse prognosis[21]. Saranath D et al have depicted a 92.0% concordance between tissues showing p53 missense mutation and overexpression of p53 protein in oral cancer and leukoplakia, thus indicating p53 immunohistochemical analysis as a reliable surrogate marker for p53 missense mutation. They have also stressed that frameshift mutation and mutation resulting in a stop codon will not be detected by immunohistochemical analysis.[22]

As p53 LI less than 25.0% was observed in 35% cases of OSCC in the study, the lower expression of p53 could be due to the rapid degradation of p53 protein by ubiquitin mediated proteolysis pathway as a result of interaction with the oncogene protein E6 of Human Papilloma Virus.[20]

In this study median p53 LI of keratosis without dysplasia was more than that of keratosis with dysplasia. This finding does not support the literature which has found increase in p53 LI with severity of dysplasia, as shown by Kurokawa H et al[9] and Nasser W et al.[19] The less number of cases of keratosis with dysplasia and moreover, all the dysplastic lesions with only mild dysplasia could be the reasons for this discrepancy. However, p53 expression in early premalignant lesions, could indicate p53 alteration as an initial event in oral carcinogenesis.[22]

In the study 50.0% cases of keratosis without dysplasia, 83.3% cases of keratosis with dysplasia and 5.9% cases of OSCC have shown Ki-67 immunostaining confined to basal and suprabasal layer. In contrast none of the cases of keratosis without dysplasia showed Ki-67 positivity in all layers of epithelium, whereas 16.7% cases of keratosis with dysplasia and 94.1% cases of OSCC have shown Ki-67 positivity in all layers of epithelium. The association of pattern of staining of Ki-67 between these three groups was statisitically significant. These findings are in agreement to similar study by Humayun S et al[3], Thomson PJ et al[23], Santos-Garcia A et al[17], Patel SM et al[15], Kurokawa H et al[9] and Iamaroon et al[16]. Dwivedi N et al[10] have shown Ki-67 positive staining confined to parabasal layers, with lack of basal staining pattern of Ki-67, however, this finding was not seen in our study.

The median Ki-67 LI increased from keratosis without dysplasia to keratosis with dysplasia to OSCC in this study, and the relationship was statistically significant. As noted in this study, similar findings were observed in the studies performed by Iamaroon et al[16], Dwivedi N et al[10], Santos-Garcia A et al[17] and Dragomir LP et al.[24] The median Ki-67 LI was also higher in keratosis with dysplasia than keratosis without dysplasia, concurrent with the findings by Kurokawa H et al[9] and Chandak AR et al.[25] The increase in number of Ki-67 positive cells with the increase in histological grades of OSCC from WDSCC to MDSCC to PDSCC has been highlighted by various authors Ashraf MJ[7], Verma R[20], Dwivedi N et al[10] and Chandak AR et al.[25] Moreover the studies done by Motta R et al[21] and Thomson PJ et al[23] have shown highest Ki-67 immunoexpression in lesion with cervical metastasis. The importance of Ki-67 overexpression in atypical oral epithelium and its association with recurrence/malignant transformation, independently of other established clinicopathological factors including age, lesion site, epithelial dysplasia has been highlighted by Yagyuu T et al in their study.[26]

A positive and insignificant correlation was observed between p53 LI and Ki-67 LI, which is in agreement with the findings by Humayun S et al[3] and Nasser W et al[19]. The authors Iamaroon A et al[16], Raju B et al[27] and Kumar P et al[28] have shown a positive and significant correlation between these the two immunomarkers. This could be explained by the fact that mutation of p53 in oral premalignant lesions and OSCC leads to uncontrolled cell proliferation, thus depicting a positive correlation between the two immunomarkers.[28]

CONCLUSION

As oral premalignant lesions have a considerable malignant proliferation, it is important not to miss these lesions in early stage and avoid false negative diagnosis. The findings in this study have confirmed the earlier reports regarding suprabasal expression of Ki67 and p53 with increasing severity of dysplasia and malignant transformation. Increased expressions of Ki-67 and p53 in OSCC compared to premalignant lesion suggest that they may be useful indicator of malignant transformation in dysplastic lesion. Similar studies with large sample size in Nepalese population can be conducted to determine the cut off value of p53 and Ki-67 LI in oral premalignant lesions and OSCC, which could aid in the early detection of these lesions.

Conflict of interests: None

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