Original Article

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Prognostic Significance of *p53*, Ki-67 and *Bcl-2* in Leukoplakia and Squamous Cell Carcinoma of the Oral Cavity

Pathology Section

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ABSTRACT

Introduction: Oral Squamous Cell Carcinoma (OSCC) is characterized by diversity in their biological behaviour which in turn forms the basis of deciding management options. It usually arises from Premalignant/Potentially Malignant Lesions (PMLs) of the oral cavity such as leukoplakias, erythroplakias, oral submucous fibrosis, lichen planus etc. Multiple summative genetic alterations occur in these lesions which result in a progression from epithelial dysplasia through in situ carcinoma to an invasive cancer.

Aim: To assess the expression of *p53*, Ki-67 and *Bcl-2* in patients having oral leukoplakia versus patients having OSCC and to correlate their immunoexpression with other clinicopathological parameters. The goal was also to determine if these biomarkers have potential as early indicators of malignancy.

Materials and Methods: This was a retrospective study which was conducted at the Department of Pathology, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India, between the period of June 2016 to September 2016. The study comprised of two groups. Group 1 included 30 histologically proven cases of oral leukoplakia and Group 2 included 30 histologically proven cases of OSCC. The OSCC cases were classified and graded on histomorphology. Both the groups were immunohistochemically analyzed for the expression of *p53*, Ki-67 and *Bcl-2* and their expression were correlated with age, sex, anatomical site, histological grading and staining intensity. The results were tabulated and statistically analyzed. A p-value less than 0.05 was considered significant.

Results: A statistically significant increase for Ki-67 expression was seen in OSCC cases than in leukoplakias while the expression of *p53* was also similar, but it was statistically insignificant. A significantly high *p53* and Ki-67 expression were seen in moderately to poorly differentiated OSCC cases. A statistically significant co-relation was also found between low immunostaining and p53 expression in the cases of leukoplakia and vice-versa in OSCC cases. No statistical significant relation was found between *Bcl-2* expression and other parameters in both the groups.

Conclusion: The present study highlights that the immunohistochemical expression of Ki-67 and *p53* should be assessed in all the cases of PMLs as these can act as predictive markers for poor prognosis in such cases. These biomarkers also allow identification of tumours with a higher rate of cell growth, thereby permitting the development of prognostic factors which effects the patient's treatment and the survival time.

Keywords: Immunohistochemistry, Oral cancer, Potentially malignant lesions, Predictive biomarkers

INTRODUCTION

Carcinoma of the oral cavity is one of the most common cancer occurring worldwide and comprise for 90% of head and neck lesions [1,2]. Annually, 3,00,000 new cases arise globally, out of which 62% occur in developing countries with the Indian sub-continent accounting for one-third of the world burden [3]. 90% of oral cancers belong to the OSCC variety [4]. OSCC is known to develop from PMLs of the oral cavity. The PMLs include variety of lesions like leukoplakias, erythroplakias, oral submucous fibrosis, lichen planus etc. Among them, leukoplakias and erythroplakias are the most common altered epithelial lesions that have a likelihood of progressing to OSCC [5].

The basis of the OSCC development is a multistep process, which is attributable to summative genetic alterations that appear very early and result in a progression from epithelial dysplasia through in situ carcinoma to an invasive disease [6,7]. These changes are in turn influenced by individual's genetic predisposition as well as by environmental influences, like tobacco, alcohol, chronic inflammation, and viral infections [8]. The most common pathophysiology behind its occurrence has also been attributed to the concept of "field-cancerization", in which there is increased risk of cancer development in the entire head and neck region due to multiple genetic abnormalities after prolonged exposure to carcinogens [9]. Tumorigenic genetic alterations includes

activation of growth promoting oncogenes, inactivation of tumour suppressor genes and alterations in the genes that regulate apoptosis, leading to unregulated cell proliferation and decreased apoptosis. These molecular changes play an integral role in cancer development and in its persistence due to genomic instability. Hence, unravelling of biological processes occurring during the preneoplastic stages is of the paramount importance, not only for the early detection of the high risk groups but also for better development of treatment strategies in this stage so as to prevent its progression to invasion [10].

The current diagnostic strategies for OSCC involves clinical and histological assessment which are highly subjective. Moreover, in patients with no clinically obvious signs of carcinoma (invasion, functional limitation, regional lymphadenopathy), diagnosing oral cancers remains a challenge. As the molecular changes occur before the histological and clinical manifestations, markers of proliferation and genomic changes could help in assessing the high risk PMLs and their potential for malignancy. In recent years, Immunohistochemistry (IHC) in oral cancers has emerged as an important tool for diagnosis and predicting prognosis, by detecting protein expression at molecular level. Various IHC markers are being used as a research tool such as *p53*, Ki-67, Epithelial Growth Factor Receptor (EGFR), *Bcl-2*, pRb, matrix metalloproteinases, CD-44, cadherins amongst others [11].

The *p53* is a tumour suppressor gene which has proved to be a important molecule in the development of many tumours [12,13]. It is located on short arm of chromosome 17 and encodes a protein of 393 amino acids. It can be inactivated through various genetic events such as mutation, loss of heterozygosity, or by epigenetic modifications such as DNA methylation or chromatin remodelling, which impairs the ability of the cells to repair and undergo apoptosis in response to DNA damage leading to uncontrolled cell growth [5,8]. *Bcl-2* is an anti-apoptotic protein located in the mitochondrial membrane, endoplasmic reticulum and nuclear membrane of a cell. Its over expression has been found in the early phase of epithelial carcinogenesis [14,15]. Ki-67 protein is a cell cycle associated human nuclear protein which is present in the peri-chromosomal region. It is specifically expressed by proliferating neoplastic cells. Cells express the antigen during G1, S, G2, and M phases but not during the resting phase G0. It is thus widely used as a proliferative marker to measure the growth fraction of cells in human cancers [16-18].

MATERIALS AND METHODS

This retrospective study was carried out in the Department of Pathology at Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India, catering to majority urban but also rural dwellers as well, from a period June 2016 to September 2016. The study was approved by research ethical committee of the institute and it comprised of two groups. Group 1 included 30 cases of histologically diagnosed oral leukoplakia and Group 2 included 30 cases of histologically diagnosed OSCC. The exclusion criteria included other premalignant lesions like submucous fibrosis, lichen planus etc., and all non squamous cell carcinomas of the oral cavity.

Detailed clinical data of the patient was noted from the records available. The sections were taken from the formalin fixed, paraffin embedded blocks of leukoplakias and OSCCs from the archives available in the department. From each paraffin embedded block, 1 section of 5 micron thickness was taken on albumin coated slide and 3 sections of 3 micron thickness were taken on poly-L-lysine coated glass slides. The first section was stained with haematoxylin and eosin (H&E). The other 3 sections were used for IHC analysis.

Antigen retrieval was done in a pressure cooker using sodium citrate buffer solution at pH 6.0. Peroxidase inhibition was then done, followed by washing in tris buffer saline, and protein block. To evaluate for *p53* expression, the primary antibody used was ready to use Mouse IgG-1 anti-*p53* (DO-7) monoclonal antibody and for Ki-67, ready to use Mouse IgG-1 anti-Ki-67 (BGX-297) monoclonal antibody; both were procured from BioGenex, Milmont drive, Fremont, California-94538 USA. For *Bcl-2*, ready to use Mouse IgG-1 anti-*Bcl-2* (100/D5) monoclonal antibody, procured from Biocare Medical, Concord, CA 94520, USA was used. Positive and negative controls were run with every batch. For secondary antibody application, slides were incubated with polymer Horseradish peroxidase reagent for 30 minutes. 3, 3-Diaminobenzidene (DAB) solution was used as the chromogen followed by counter staining with haematoxylin.

The H&E stained sections were examined under light microscopy to histologically grade OSCC as Well Differentiated (WDSCC), Moderately Differentiated (MDSCC), and Poorly Differentiated (PDSCC). Among the immunohistochemically stained sections, 500 cells were counted under 40X power of microscope in each slide and the percentage of positive cells was noted. For evaluating *p53* and Ki-67 expression, the epithelial cell nuclei with clear brown colour, were regarded as positive. For *Bcl-2*, cytoplasmic light brown staining in the epithelial cells was considered as positive, while the staining of lymphocytes acted as an internal control.

Scoring for *p53*, Ki-67 and *Bcl-2* staining intensity was done. A score of 0, 1+ and 2+ was given to slides that were negative or with fewer than 5% positive cells, showed positive immunoreaction in 6-25% cells and showed positive immunoreaction in 26-100% of cells, respectively.

The distribution of the staining was recorded depending on whether the staining was confined only to the basal and parabasal layers, or present in all the layers of the epithelium. The clinical records corresponding to the cases were used to gather data on age, sex and the pathological characteristics of the lesions, such as their localization, stage, and degree of differentiation.

STATISTICAL ANALYSIS

The results were tabulated and subjected to statistical analysis using Microsoft Excel 2013 and SPSS version 16.0. The relationship between IHC expression and clinicopathological parameters was compared using Chi square test. A p-value less than 0.05 was considered significant.

RESULTS

In the study Group 1, out of the 30 cases of leukoplakia, most of the lesions were diagnosed in patients aged less than 40 years (72%), while in the study Group 2, i.e., of 30 OSCC cases, the mean age of the patients was 45.17 years. Male preponderance was seen in both the groups [Table/Fig-1]. Most common site of OSCC was buccal mucosa (36.7%) followed by the tongue (33.3%). Among OSCC, 8 (26.7%) were WDSCC, 20 (66.7%) were MDSCC and 2 (6.7%) were PDCC [Table/Fig-2].

Of the total 30 leukoplakia cases, *p53* positive expression was seen in 15 cases (50%), Ki-67 positive expression was seen in 14 cases (46.6%) and *Bcl-2* positivity was evident in 19 cases (63.3%). This expression was confined to basal and parabasal layer. Out of the 30 OSCC cases, the expression of *p53* was found to be positive in 21 cases (70%) while Ki-67 and *Bcl-2* positive expression was seen in 26 cases (86.6%) and 19 cases (63.3%) respectively. The expression of these markers was randomly expressed in all layers in OSCC [Table/Fig-3,4]. On comparing the 2 groups, *p53* expression was slightly more in OSCC cases than in leukoplakia cases, although this was statistically insignificant while Ki-67 expression was significantly higher in OSCCs than in leukoplakias (p=0.001). *Bcl-2* positive expression was equal in both the groups and was statistically insignificant.

In leukoplakia, all *p53* positive cases showed low immunostaining (i.e., 6-25% cells) while among OSCCs, most cases had 26-100% immunopositive cells. This finding was statistically significant (p=0.0001). In OSCC cases, *p53* was significantly more expressed in MDSCC to PDSCC than in WDSCC (p=0.039). Ki-67 was also associated with a poorer degree of differentiation (p=0.012). No association between *Bcl-2* expression and degree of differentiation was found [Table/Fig-5].

No statistical significant findings were found among expression of all these three markers and age, gender and location of the lesions (p>0.05).

Demographic Parameters		Leukoplakia		OSCC	
		Frequency (n=30)	Percentage (%)	Frequency (n=30)	Percentage (%)
Age	<40 years	15	50	11	36.7
	40-60 years	12	40	14	46.7
	>60 years	3	10	5	16.7
Sex	Male	25	83.3	27	90.0
	Female	5	16.7	3	10.0

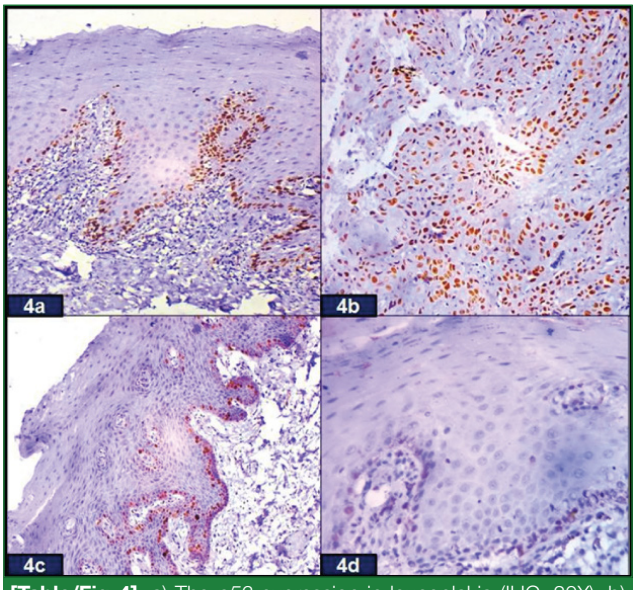
[Table/Fig-1]: Table showing age and sex preponderance of leukoplakia versus OSCC cases.

OSCC		Frequency (n=30)	Percentage (%)
Localisation	Buccal Mucosa	11	36.7
	Tongue	10	33.3
	Soft Palate	3	10
	Tonsillar Fossa	2	6.7
	Angle of Mouth	1	3.3
	False Vocal Cord	1	3.3
	Hard Palate	1	3.3
	Lower Lip	1	3.3
Degree of Differentiation	Well differentiated	8	26.7
	Moderately differentiated	20	66.7
	Poorly differentiated	2	6.7

[Table/Fig-2]: Table showing buccal mucosa as the most common site and moderately differentiated SCC as major degree of differentiation in OSCC.

Immune Markers	Leukoplakian (%)	OSCC n (%)	p-value
p53	15 (50)	21 (70)	0.147
Bcl-2	19 (63.34)	19 (63.34)	0.605
Ki-67	14 (46.66)	26 (86.66)	0.001

[Table/Fig-3]: Comparison of biomarkers positive expression between leukoplakia and OSCC.
*(n=30)



[Table/Fig-4]: a) The *p53* expression in leucoplakia (IHC, 20X); b) The *p53* stained section showing positive result in MDSCC of the oral cavity (IHC, 20X); c) Parabasal and basal expression of Ki-67 in leukoplakia (IHC, 20X); d) *Bcl-2* expression in leukoplakia (IHC, 40X).

Expression of Biomarkers	Percentage (%) Positive Cells	Grade of OSCC		p-value
		Well Differentiated	Moderate-Poorly Differentiated	
p53	Negative	3	7	0.039
	Positive in 6-25% cells	3	1	
	Positive in 26-100% cells	2	14	
Bcl-2	Negative	3	3	0.278
	Positive in 6-25% cells	5	14	
	Positive in 26-100% cells	0	0	
Ki-67	Negative	2	2	0.012
	Positive in 6-25% cells	4	2	
	Positive in 26-100% cells	2	18	

[Table/Fig-5]: Table showing statistically significant expression of *p53* and Ki-67 in MDSCC and PDSCC of the oral cavity.

DISCUSSION

There is a worldwide escalation in number of patients having OSCC, according to World Health Organization [19]. Therefore, it is an ideal candidate for screening not only because of its evidently high burden, but also because 90% of cases occurring globally have been attributed to tobacco (chewing/smoking) and alcohol consumption, which are preventable risk factors [20,21]. Moreover, it has clinically distinguishable PMLs that can aid in early diagnosis and management.

The molecular medicine aims at the development of diagnostic procedures to accurately predict the response to therapeutic agents. Since, OSCC is thought to develop from precancerous dysplastic lesions by multistep carcinogenesis which is under genetic control, it is possible that the genomic markers like *p53*, *Bcl-2* and Ki-67 might help in assessing the malignant potential of PMLs. In such cases, IHC seems as an optimal technique for such an assessment at molecular level that could be easily and routinely incorporated in clinical practice.

In the present study, the mean age of leukoplakia was 40.67 years and mean age of OSCC was 45.17 years. Most OSCC cases were diagnosed in 5th and 6th decade of life. Both conditions affected males more commonly than females (5.2:1 in leukoplakias and 9:1 in OSCCs), which could be attributable to the higher consumption of tobacco and alcohol among men. Most common site of OSCC was buccal mucosa (36.67%), followed by tongue, possibly due to the habit of tucking the betel quid while chewing. These findings were similar to the results of studies done by Sharma P et al., [22] and Shenoi R et al., [23].

Ki-67 and *p53* positive expression was detected in 86.6% and 70% cases of OSCC respectively, which is in accordance with other studies [24-26]. In comparison to leukoplakias, *p53* expression was higher in OSCC, though it was not statistically significant, while Ki-67 expression was statistical significantly higher in OSCC cases than leukoplakias. A previous study in 14 OSCC cases with tobacco and betel quid chewing habits found that the expression of *p53* as well as Ki-67 significantly increases as normal oral mucosa becomes dysplastic and undergoes malignant transformation [4,25] and the significantly higher expression of *p53* in OSCC cases in that study could be explained by the fact that they included only cases with tobacco and betel chewing habits whereas, it was not so in the present study. Another study similarly concluded that increased Ki-67 expression was associated with high grade dysplasia [24].

The present study showed that in leukoplakias, all *p53* positive cases showed a statistically significant low staining (i.e., 6-25% cells were positive) in contrast to OSCCs, where most cases had 26-100% immunopositive cells. This finding was in accordance with the study done by Humayun S et al., [4], where the percentage of *p53* positive cells in normal mucosa was 15-25% which was increased to 95% in malignant mucosa. Thus, high *p53* expression (>25% positivity) can be used as predictive marker of OSCC transformation in PML cases. However, no correlation was found between Ki-67 expression and the percentage of immunopositive cells.

The *p53* and Ki-67 expression in leukoplakia cases was confined to the basal layer whereas OSCC cells showed random expression in all layers of the epithelium. Similar findings have been reported by other authors [4,25]. Few researchers have also documented that 86% of premalignant lesions that showed *p53* expression above the basal layer developed into OSCC [27].This suggests that suprabasal expression of *p53* could be an early event in oral carcinogenesis and can be used as a predictive marker of a developing carcinoma. Torres-Rendon et al., [28], have reported that for Ki-67, the expression is mainly confined to parabasal compartment of normal oral mucosa as most of the cells are in the G0 phase with very less number of cells in a G0-G1 transition and its expression becomes random in all layers as one progresses from a PML to OSCC as number of cells increase in a cell cycle during progression.

On comparing the *p53* and Ki-67 expression with the histological grade, it was found that the both these markers were significantly more expressed in moderate to poorly differentiated cases than in well-differentiated cases (p=0.039 and p=0.012 respectively). A previous study, found similar statistical significant correlation among *p53* expression and the degree of malignancy, with higher *p53* expression in poorly differentiated cases [4]. Similarly, for Ki-67, many authors have documented that Ki-67 overexpression is highest in PDSCC when compared to WDSCC and MDSCC cases [16,24,25,29]. Kannan S et al., [30], also reported that poorly differentiated tumours had prominent alterations in

Ki67 and *p53* expression than well differentiated ones.

63% OSCC cases were *Bcl-2* positive, compared to 51.6% cases in a previous study [31]. It is believed that *Bcl-2* plays a role in relatively early stages of carcinogenesis and that positive *Bcl-2* expression may be an indicator of poor prognosis in oral cancer. The present study found no association between *Bcl-2* expression and malignant transformation as its expression was equal in both the leukoplakia and OSCC cases. Also, no association was found between *Bcl-2* and the percentage of immunopositive cells and the grade of malignancy. This finding in our study was in disconcordance with study done by Singh BB et al., [32], who have reported that there is a down regulation of *Bcl-2* expression during progression from oral epithelial dysplasia to OSCC while in another selective prospective study of 30 OSCC cases and 18 precancer cases done by Arya V et al., [15], it was found that there was a statistically significant difference in the *Bcl-2* expression between oral cancer and pre cancer state with p-value of <0.01. Nevertheless, Leyva-Huerta ER et al., [33], have reported in their study that *Bcl-2* expression was negative in all leukoplakia and OSCC cases, though mild positivity was observed in normal tissue.

LIMITATION

A large sample size along with the response of patients to therapy could have given better results.

CONCLUSION

Higher *p53* expression (>25% positivity) and suprabasal expression of *p53* can be a predictive marker for poor prognosis in PMLs cases. The expression of both *p53* and Ki-67 is associated linearly with the decrease of the degree of tumour differentiation and with high degree dysplasia. Thus, the results emphasize that taken along with Ki-67, *p53* will be of great value as an adjuvant in assessing malignant potential of PMLs, however, *Bcl-2* is of less utility for such an evaluation. Therefore, we recommend including *p53* and Ki-67 immunohistochemical analysis for diagnosing high risk PMLs so that early diagnosis and intervention is possible, thereby improving the treatment outcome and patient survival.

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