Original Article

Expression of p-16, Ki-67 and p-53 markers in dysplastic and malignant lesions of the oral cavity and oropharynx

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Abstract

Background: Understanding the markers for predicting degree of dysplasia and progression to malignancy can help early identification and prompt treatment of patients with oral cancers. In this study, we aim to identify and characterize different tumor suppressor genes such as p-53 and p-16 and proliferation marker Ki-67 in defining stages of dysplasia of oral mucosa and grading of tumor.

Settings and Design: Oral biopsy tissues (for neoplastic lesions) received for histopathological evaluation were included in the study. The sections were processed for H&E staining, and 112 cases were chosen for immunohistochemical study. The data were analyzed by Chi-square and *z*-tests using software SPSS.

Results: We found significant correlation between degree of dysplasia and p-16 immunoexpression with 16.7% of cases showing positivity in oral intraepithelial neoplasia (OIN) I cases as compared to 25% in OIN II and 77.8% in OIN III. Ki-67 immunoexpression correlated significantly with both histological type and grade of tumor with increased expression and intensity seen in malignant cases (66.3%) as compared to benign (10%) and premalignant cases (37%) and higher Ki-67 immunoexpression in poorly differentiated tumors (75%) than well-differentiated tumors (12.2%). Regarding p-53 immunoexpression, positive staining was seen in only malignant cases and premalignant cases.

Conclusions: Ki-67 and p-16 can be useful as a marker of degree of dysplasia and transformation to malignancy. Ki-67 can also serve as a marker of degree of differentiation of tumors. Hence, they can serve as important ancillary markers to analyze the transition to carcinoma, dysplasia and progression of tumor.

Keywords: Dysplasia, Ki-67, oral squamous oral carcinoma, p-16, p-53

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INTRODUCTION

In India, oral cancer is among the top three types of cancers with 4 out of 10 of all cancers being oral cancers, hence constituting a major public health problem.[1] The problem is even alarming in central Indian regions like Bhopal due to high prevalence of tobacco usage in this population.[2] Understanding the markers for predicting degree of dysplasia and progression to malignancy can help early identification and prompt treatment of patients with oral cancers.

Oral carcinogenesis is a multistage process arising from the accumulation of genetic events that disturb cell cycle control, proliferation, motility, survival and tumor-related angiogenesis.[3] Recent studies have

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hypothesized that inactivation of some tumor suppressor genes, namely p-53 (tumor protein 53) and p-16 (CDKN2A cyclin-dependent kinase inhibitor 2A), may play a significant role in oral carcinogenesis.[4,5] In addition, proliferation markers such as Ki-67 marker have been examined in oral epithelial dysplasia and oral squamous cell carcinoma.[6]

In this study, we aim to document the status of p-16, p-53 and Ki-67 expression in nonneoplastic, dysplastic and malignant oral lesions and to establish if any correlation exists between the localization and intensity of expression of p-16, p-53 and Ki-67 staining pattern and the degree of dysplasia, histological type and tumor grade.

SUBJECTS AND METHODS

Cases

This study was conducted in our institute between the duration March 1, 2017–July 1, 2018. All the oral biopsy tissues (for neoplastic lesions) received in the department of pathology for histopathological evaluation during the study duration, were included in the study. Biopsies with tissue insufficient for histopathological evaluation and autolyzed samples were excluded from the study. The study was approved by institutional ethics committee. A thorough history Information was taken from requisition forms received in department of pathology. History was also taken through interview and case files. All the biopsy samples were processed for H&E staining, and slides were evaluated by two investigators independently. The reporting was done using Broder's grading system[7] and WHO tumor classification.[8]

Immunohistochemistry

112 cases were included which comprised of 12 benign cases, 19 premalignant cases (six OIN 1, four OIN II and nine OIN III), 41 cases of well-differentiated squamous cell carcinomas (WDSCC), 31 cases of moderately differentiated SCC (MDSCC), 8 cases of poorly differentiated SCC (PDSCC) and 3 cases of verrucous carcinoma. The selected sections were mounted on poly-L-lysine-coated slides. Immunohistochemistry (IHC) was performed using antibodies Ki-67 (Clone SP6, dilution 1:200, Thermo Fisher Scientific, UK), p-53 (clone DO-7, dilution 1:200, Thermo Fisher Scientific, UK) and p-16 (clone 1E12E10, Thermo Fisher Scientific, UK) as indicated on Pierce™ Peroxidase IHC Detection Kit by Thermo Fisher Scientific. The results were recorded as index of positivity (IP) score and staining intensity in accordance with previous studies.[9,10] IP score was assigned 0 (<10%), 1 (10%–50%) and 2 (>50%) according to the percentage of positive stained cells in 1000 counted cells in the ×40 microscope field. Only a score of 1 or 2 was considered as positive immunoexpression. 0 score was considered as negative. For p-53 and Ki-67, nuclear positivity was considered as positive expression, and for p-16, combined nuclear and cytoplasmic staining was considered as positive expression. Intensity of staining was scored as 0 (weak), 1 (moderate) and 2 (strong).[9,10]

Statistics

The data were analyzed using appropriate statistical tests using software SPSS (IBM, SPSS Statistics, version 20). The qualitative data were expressed in terms of percentages. Comparison of the qualitative variables between groups was done using the Chi-square test as well as *Z* test. *P* value was considered significant if $P < 0.05$ and highly significant if $P < 0.01$. *Z* score >1.96 was considered significant.

RESULTS

p-16 immunoexpression

Irrespective of the histological type/grade, 35.7% of total cases showed positive immunoexpression with diffuse positivity (IP score of 2) in 23.2% of cases [Figure 1]. Benign cases, which were all chronic inflammation cases, did not show any positive p-16 immunoexpression. Premalignant lesions had higher proportion of cases with

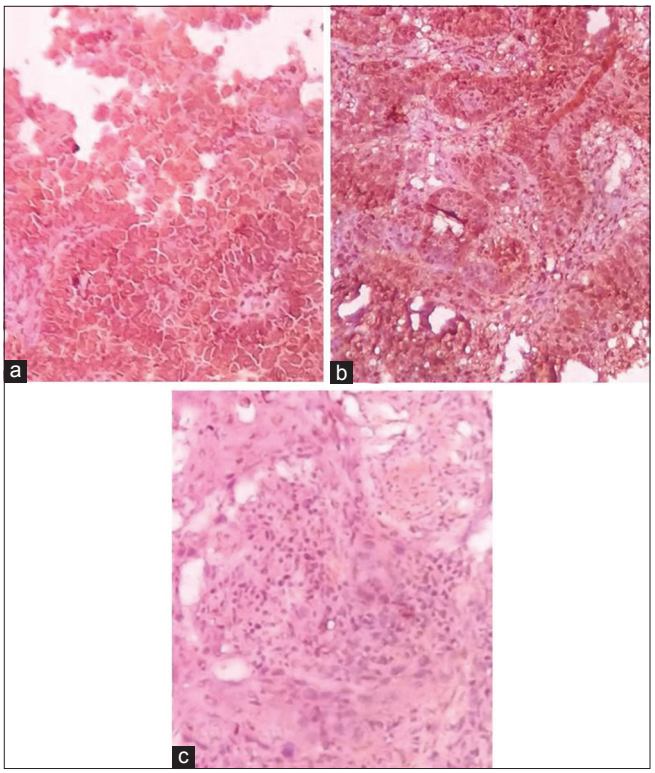

Figure 1: Representative immunohistochemical patterns of p-16 (a) strongly positive immunoexpression (IP score 2) in a case of poorly differentiated SCC, (b) strongly positive immunoexpression in a case of moderately differentiated SCC showing IP index 2, (c) negative immunoreactivity in a case of moderately differentiated SCC. SCC: Squamous cell carcinomas, IP: Index of positivity

positive p-16 immunoexpression (47.4%) than the malignant cases (37.3%); however, the difference was not statistically significant. Among the premalignant cases, OIN I lesions show positive p-16 immunoexpression in only 16.7% of cases (all with IP score of 1) as compared to 25% in OIN II and 77.8% in OIN III. Hence, the positivity increased with increase in degree of dysplasia with severe dysplasia (OIN III) cases displaying 77.8% of positivity (44% diffuse positivity) (Chi-square – 10.8; $P = 0.03$).

Among the various grades of squamous cel l carcinoma, maximum proportion of p-16 immunoexpression positive cases were found in poorly differentiated carcinoma (75%), followed by moderately differentiated carcinoma (35.5%) and well-differentiated carcinomas (29.3%). However, the difference in immunoexpression between these different grades of oral SCC is not statistically significant [Table 1].

Ki-67 immunoexpression

Only one case of benign chronic inflammation histology showed positive Ki-67 immunoexpression. About 37% of premalignant cases (7 of 19 cases) displayed positive immunoexpression with IP score of 1 in 15.8% of cases and IP score of 2 in 21.1% of cases. In contrast, among malignant cases, significantly higher proportions of cases (66.3%) displayed positive immunoexpression (Chi-square – 15.4; $P = 0.004$) [Figure 2]. Among the malignant cases, PDSCC and MDSCC cases had significantly higher proportions of cases exhibiting diffuse positivity (75% and 41.9%, respectively) as compared to WDSCC cases (12.2%) (Chi-square – 39.09; $P = 0.0001$). Among the premalignant cases, positive Ki-67 immunoexpression, i.e., suprabasal expression was seen exclusively in OIN III cases with 44.3% and 33.3% of cases displaying IP score of 2 and 1, respectively [Table 2].

Ki-67 intensity scores followed similar trend with a strong intensity of staining seen in significantly higher number of malignant cases (60.2%) than benign cases and premalignant

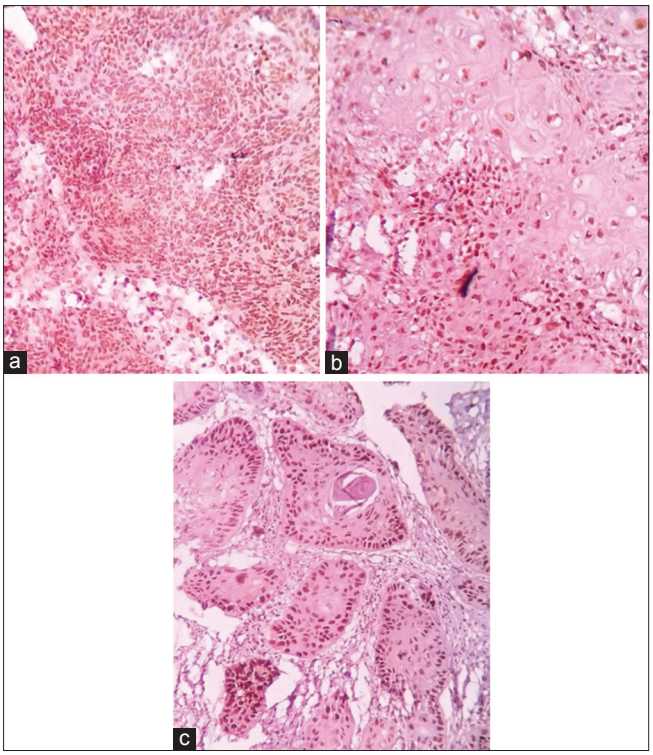

Figure 2: Immunohistochemical findings of Ki-67 expression (a) strongly positive immunoexpression in a case of poorly differentiated SCC (IP index 2), (b) well-differentiated SCC showing IP index 0, (c) moderately differentiated SCC showing positive immunoexpression (IP score of 1). SCC: Squamous cell carcinomas, IP: Index of positivity

Table 1: p-16 index of positivity score and p-16 intensity score in relation to (a) benign, premalignant and malignant lesions and (b) specific histotypes

(a) Benign, premalignant and malignant lesions

Histotype	Number of cases with p16 IP score (%)			Total	Number of cases with p16 intensity score (%)		
	0	1	2		0	1	2
Benign	10 (100.0)	0 (0.0)	0 (0.0)	10	10 (100.0)	0 (0.0)	0 (0.0)
Premalignant	10 (52.6)	4 (21.1)	5 (26.3)	19	11 (57.9)	2 (10.5)	6 (31.6)
Malignant	52 (62.7)	10 (12.0)	21 (25.3)	83	49 (59.0)	9 (10.8)	25 (30.1)
Total	72 (64.3)	14 (12.5)	26 (23.2)	112	70 (62.5)	11 (9.8)	31 (27.7)

(b) Specific histotypes

Histotype	Specific Histotypes	Number of cases with p16 IP score			Total	Number of cases with p16 intensity score		
		0	1	2		0	1	2
Benign	CI	10 (100.0)	0 (0.0)	0 (0.0)	10	10 (100.0)	0 (0.0)	0 (0.0)
Premalignant	OIN I	5 (83.3)	1 (16.7)	0 (0.0)	6	5 (83.3)	1 (16.7)	0 (0.0)
	OIN II	3 (75.0)	0 (0.0)	1 (25.0)	4	3 (75.0)	0 (0.0)	1 (25.0)
	OIN III	2 (22.2)	3 (33.3)	4 (44.4)	9	3 (33.3)	1 (11.1)	5 (55.6)
Malignant	WDSCC	29 (70.7)	4 (9.8)	8 (19.5)	41	27 (65.9)	3 (7.3)	11 (26.8)
	MDSCC	20 (64.5)	5 (16.1)	6 (19.4)	31	19 (61.3)	5 (16.1)	7 (22.6)
	PDSCC	2 (25.0)	1 (12.5)	5 (62.5)	8	2 (25.0)	1 (12.5)	5 (62.5)
	Verrucous	1 (33.3)	0 (0.0)	2 (66.7)	3	1 (33.3)	0 (0.0)	2 (66.7)

IP: Index of positivity, OIN: Oral intraepithelial neoplasia, WDSCC: Well-differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous-cell carcinoma, CI: Chronic inflammation

cases (10% and 36.8%, respectively) (Chi-square – 25.0; $P = 0.0001$). Among the malignant cases, stronger intensity of staining was seen in 65.9% of WDSCC cases, 58.1% of MDSCC cases and 25% of PDSCC cases [Table 2].

p-53 immunoexpression

p-53 immunoexpression was positive in 42.1% of malignant cases and 42.2% of premalignant cases [Figure 3]. None of the benign cases showed positive immunoexpression for p-53. Malignant cases exhibited an IP score of 1 in 30.1% of malignant cases and IP score of 2 in 12% of malignant cases. Premalignant cases showed an IP score of 1 and 2 in 21.1% cases each. Among the malignant cases, positive p-53 immunostaining were seen in 55% of MDSCC cases as compared to 39% of WDSCC cases and 25% PDSCC cases. In premalignant group, 55.5% of OIN III cases show positive suprabasal immunoexpression as compared to 0% of OIN II cases and 50% of OIN I cases [Table 3].

Intensity of staining of p53 was mostly weak to moderate with strong intensity seen in 35.5% cases of MDSCC as compared to 12.5% cases of PDSCC and 22.2% cases of WDSCC. Among premalignant cases, 44.4% of OIN III cases showed strong intensity of staining and 22.2% moderate intensity of staining. About 33.3% of cases of OIN II show moderate staining intensity and 33.3% of cases show strong staining intensity.

Correlation between expressions of different immunohistochemistry markers

Analysis for correlation between Ki-67 and p-53 index exhibited a pattern of higher p53 index 2 (70%) in cases with higher Ki-67 score of 2 (Pearson Chi-square – 13.7; $P = 0.008$) [Figure 4].

DISCUSSION

The current study was done to assess if histological diagnostic modality can be aided by immunohistochemical markers such as cell-cycle proteins p-16 and p-53, together with the proliferation index marker Ki-67 in predicting the evolution of benign into invasive carcinomas of the oral cavity and oropharynx and in grading the tumors.

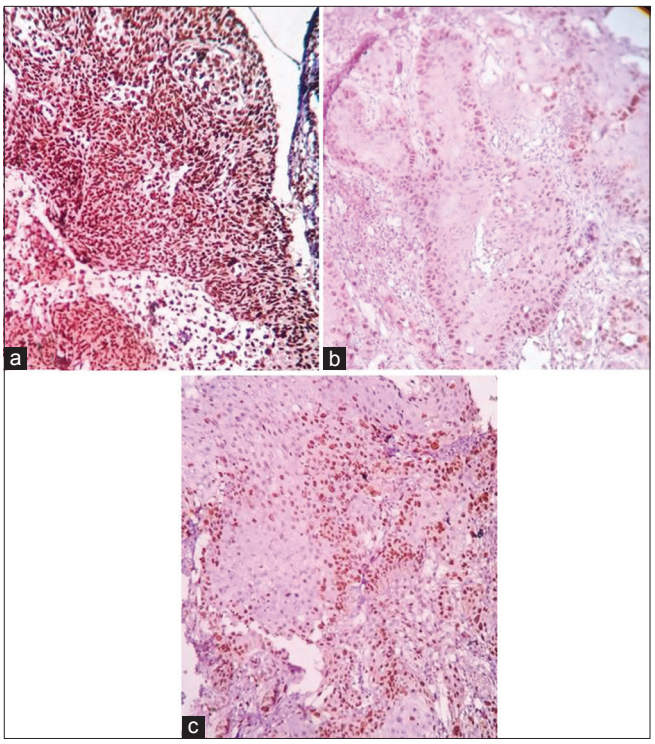

Figure 3: p-53 staining in (a) moderately differentiated SCC showing IP index 2, (b) well-differentiated SCC showing IP index 0, (c) moderately differentiated SCC showing IP index 1. SCC: Squamous cell carcinomas, IP: Index of positivity

Table 2: Ki-67 index of positivity score and Ki-67 intensity score in relation to (a) benign, premalignant and malignant lesions and (b) specific histotypes

(a) Benign, premalignant and malignant lesions								
Histotype		Number of cases with Ki-67 IP score (%)			Total	Number of cases with Ki-67 intensity score (%)		
		0	1	2		0	1	2
Benign		9 (90.0)	1 (10.0)	0 (0.0)	10	7 (70.0)	2 (20.0)	1 (10.0)
Premalignant		12 (63.2)	3 (15.8)	4 (21.1)	19	8 (42.1)	4 (21.1)	7 (36.8)
Malignant		28 (33.7)	31 (37.3)	24 (28.9)	83	9 (10.8)	24 (28.9)	50 (60.2)
Total		49 (43.8)	35 (31.3)	28 (25.0)	112	24 (21.4)	30 (26.8)	58 (51.8)
Histotype	Specific Histotypes	Number of cases withKi-67 IP score			Total	Number of cases with Ki-67 intensity score		
		0	1	2		0	1	2
Benign	CI	9 (90.0)	1 (10.0)	0 (0.0)	10	7 (70.0)	2 (20.0)	1 (10.0)
Premalignant	OIN I	6 (100.0)	0 (0.0)	0 (0.0)	6	4 (66.7)	2 (33.3)	0 (0.0)
	OIN II	4 (100.0)	0 (0.0)	0 (0.0)	4	4 (100.0)	0 (0.0)	0 (0.0)
	OIN III	2 (22.2)	3 (33.3)	4 (44.4)	9	0 (0.0)	2 (22.2)	7 (77.8)
Malignant	WDSCC	19 (46.3)	17 (41.5)	5 (12.2)	41	5 (12.2)	9 (22.0)	2 (65.9)
	MDSCC	8 (25.8)	10 (32.3)	13 (41.9)	31	4 (12.9)	9 (29.0)	18 (58.1)
	PDSCC	1 (12.5)	1 (12.5)	6 (75.0)	8	0 (0.0)	6 (75.0)	2 (25.0)
	Verrucous	0 (0.0)	3 (100.0)	0 (0.0)	3	0 (0.0)	0 (0.0)	3 (100.0)

IP: Index of positivity, OIN: Oral intraepithelial neoplasia, WDSCC: Well-differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, CI: Chronic inflammation

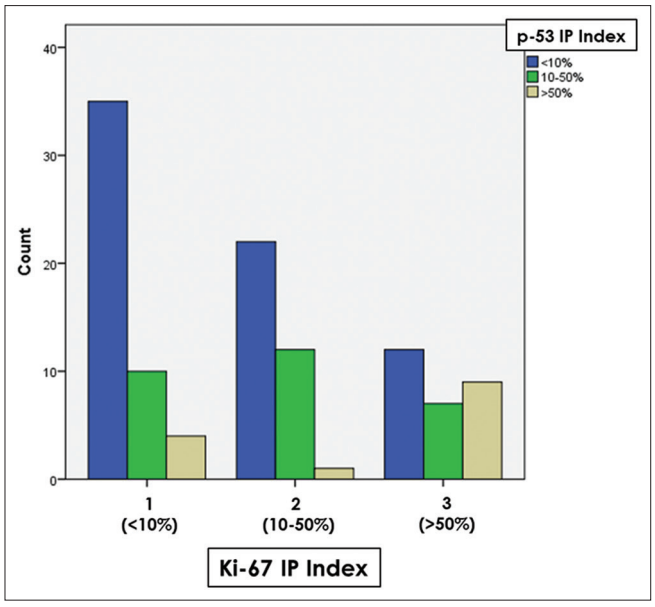

Figure 4: Correlation between p-53 IP index and Ki-67 IP index of studied cases. IP: Index of positivity

In our study, the p-16 immunoexpression was identified in 47.4% of the premalignant cases and 37.3% of malignant cases with maximal positivity in OIN III lesions (77.8%). Hence, we found increased immunoexpression with increasing grades of dysplasia with OIN I lesions showing positive p-16 immunoexpression in only 16.7% of cases as compared to 25% in OIN II and 77.8% in OIN III. Majority of malignant lesions displayed lower p-16 immunoexpression than OIN III lesions. Only the poorly differentiated tumors had high proportion of cases with positive p-16 immunostaining. This is in contrast to other studies who have found low to absent p-16 immunoexpression at the level of the invasion front of poorly differentiated tumors.[11,12] Similar to our study, Angiero *et al.* have demonstrated an increase in p-16 expression in higher grades of dysplasia and invasive oral squamous cell tumors.[13] Dragomir *et al.*, 2012[14] and Gologan *et al.*[15] found that the positivity index and the intensity of reaction were increased at the level of dysplastic epithelium for p-16 but reduced in tumor and their invasion front. Still, others have not found any correlation between p-16 immunoexpression and tumoral grade and stage.[16,17] Although the literature shows conflicting results for oral lesions without any consensus, our present study infers that p-16 immunoexpression and pattern correlates with the malignant transformation in oral and oropharyngeal lesions with increased immunoexpression in increasing degrees of dysplasia.

p-16 expression with diffuse positivity in more than 70% of cells has been suggested as a surrogate marker of active high-risk human papillomavirus (HPV) oncogene expression in oral and oropharyngeal carcinomas.[18,19] HPV prevalence in SCC of the oral cavity and oropharynx in India has been reported to be ranging from 15% to 51%.[20,21] In our study, irrespective of the histology, 23.2% of cases displayed a diffuse positivity. Considering p-16 as a surrogate marker of HPV, our study points to a prevalence of HPV to be around 23.2% which corresponds well with the above studies. The etiologic and prognostic role of HPV in oropharyngeal cancers is well established with HPV tumor positivity favorably influencing outcome, including overall survival, disease-free survival and recurrence.[22] However, our study lacked the variables to assess p-16 immunoexpression as a prognostic marker. Further studies are warranted to make such assessment.

In our study, increased immunoexpression of Ki-67 was found in malignant cases as compared to premalignant and benign cases. Only one benign case showed positive Ki-67 activity possibly due to prominent chronic inflammation due to proliferation of lymphocytes. Among the malignant cases, significantly higher immunoexpression was found in poorly differentiated squamous cell carcinoma cases as compared to well-differentiated squamous cell carcinoma. Intensity of staining of Ki-67 followed similar trend with significantly stronger intensity of staining malignant cases than benign cases and premalignant cases. Among premalignant cases, positive Ki-67 expression was only seen in OIN III cases. The results are in accordance to data in the literature, Ki-67 proving to be useful in assessing tumor aggressiveness.[10,14] Ki-67 is a nuclear protein expressed in the G2- and M-phases of actively dividing cells.[23] Increased cellular proliferation is associated with more advanced lesions, and the distribution of proliferating cells in tissues may tell us more about the regulatory mechanisms that become dysfunctional during carcinogenesis.[23]

p-53 immunoexpression was detected in 42% of malignant cases as compared to no immunoexpression in benign cases which is in accordance to the results described in the literature.[24,25] Although we detected more cases of p-53-positive MDSCC compared to WDSCC and PDSCC, this difference was not significant. Previous reports are also inconclusive when relating p-53 immunoexpression with the differentiation grade of oral SCC.[12,17] It has been suggested that p-53 expression in oral SCC apparently does not correlate with differentiation grade but has been associated with poor patient outcome.[26,27]

In premalignant lesions, a suprabasal p-53 immunohistochemical staining has been considered to be predictive for malignant transformation and progression

Table 3: p-53 index of positivity score and p-53 intensity score in relation to (a) benign, premalignant and malignant lesions and (b) specific histotypes

(a) Benign, premalignant and malignant lesions							
Histotype	Number of cases with p-53 IP score			Total	Number of cases with p-53 intensity score		
	0	1	2		0	1	2
Benign	10 (100.0)	0 (0.0)	0 (0.0)	10	10 (100.0)	0 (0.0)	0 (0.0)
Premalignant	10 (52.6)	4 (21.1)	5 (26.3)	19	11 (57.9)	2 (10.5)	6 (31.6)
Malignant	52 (62.7)	10 (12.0)	21 (25.3)	83	49 (59.0)	9 (10.8)	25 (30.1)
Total	72 (64.3)	14 (12.5)	26 (23.2)	112	70 (62.5)	11 (9.8)	31 (27.7)

(b) Specific histotypes								
Histotype	Specific Histotypes	Number of cases with p-53 IP score			Total	Number of cases with p-53 intensity score		
		0	1	2		0	1	2
Benign	CI	10 (100.0)	0 (0.0)	0 (0.0)	10	9 (90.0)	1 (10.0)	0 (0.0)
Premalignant	OIN I	3 (50.0)	1 (16.7)	2 (33.3)	6	4 (66.7)	0 (0.0)	2 (33.3)
	OIN II	4 (100.0)	0 (0.0)	0 (0.0)	4	3 (75.0)	1 (25.0)	0 (0.0)
	OIN III	4 (44.4)	3 (33.3)	2 (22.2)	9	3 (33.3)	2 (22.2)	4 (44.4)
Malignant	WDSCC	25 (61.0)	13 (31.7)	3 (7.3)	41	19 (46.3)	13 (31.7)	9 (22.0)
	MDSCC	14 (45.2)	12 (38.7)	5 (16.1)	31	10 (32.3)	10 (32.3)	11 (35.5)
	PDSCC	6 (75.0)	0 (0.0)	2 (25.0)	8	6 (75.0)	1 (12.5)	1 (12.5)
	Verrucous	3 (100.0)	0 (0.0)	0 (0.0)	3	0 (100.0)	0 (0.0)	2 (0.0)

IP: Index of positivity, OIN: Oral intraepithelial neoplasia, WDSCC: Well-differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, CI: Chronic inflammation

to oral squamous carcinoma cases.[10,13,17] We found positive staining pattern in around 42% of premalignant cases, with OIN III cases showing 55.5% positivity. However, around 50% of OIN I cases also exhibited positive p-53 expression. This is in agreement with other studies[12] suggesting that p-53 could be not sensitive enough to predict which precancerous lesions will progress to cancer.[28]

A positive significant correlation was observed between positivity index of p-53 and Ki-67 which indicates that there is increase in p-53 immunoexpression with increase in Ki-67 immunoexpression in malignant lesions. Motta *et al*. 2009[24] also found significant coexpression of these two markers which was related to larger tumors, metastasis to lymph nodes and very likely to a worse prognosis. Lavertu *et al*. 2009[29] stated that the coexpression of p-53 and Ki-67 markers is associated with a lower time of survival free from disease attributed to both neoplasia recurrence and/or an appearance of a second early primary site. Hence, this subset of tumors with coexpression of higher positivity index of p-53 and Ki-67 may represent tumors with poor prognosis. As we did not follow the patients prospectively and did not take into account tumor size and lymph node involvement, we cannot assess them as prognostic markers.

CONCLUSIONS

To conclude, we found that Ki-67 and p-16 can be useful as a marker of degree of dysplasia and transformation to malignancy. Ki-67 in addition can also serve as a marker of degree of differentiation of tumors. p-53 did not show any conclusive results; however, it can serve as a marker suggesting transition from chronic inflammation to dysplasia/carcinoma. Hence, Ki-67, p-16 and p-53 can serve as important ancillary markers to analyze the transition, degree of dysplasia and progress of tumor with respect to clinicopathological parameters. Thus, these immunohistochemical marker panels could be integrated with clinicopathological parameters for better assessment of patients with oral lesions. They can aid to detect early lesions and thus may improve overall prognosis. We recommend further evaluation of these markers for prognostic assessment. Such information will further enhance the judgment of clinicians for deciding the treatment protocol in the interest of the patients.

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Conflicts of interest

There are no conflicts of interest.

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