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Original Research Article

Study of P16 and Ki-67 Immunoexpression in Squamous Cell Neoplastic Lesions of Oropharynx and Larynx

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Abstract

Background: The cancerous lesions of head and neck region show diverse clinicopathological features and are mostly linked with poor outcome. Aim: To evaluate the p16 and Ki-67 expression in benign lesions, dysplastic lesions and squamous cell carcinoma (SCC) of oropharyngolaryngeal (OPL) region.

Materials and Methods: 62 histopathologically diagnosed cases were taken from the pathology department of our institute. It comprised of 6 benign cases, 12 dysplasia cases and 44 SCC cases of OPL region. Immunohistochemistry was done to investigate the expression of p16 and Ki-67. The scoring was done based on percentage of positive tumor cells and the staining intensity.

Results: Out of 62 cases, 39 cases(62.9%) showed p16 expression and 58 cases(93.5%) showed Ki-67 expression. There was significant increase in staining intensity and percentage of tumor cells expressing p16 and Ki-67 from benign lesion to dysplasia to different grades of SCC.

Conclusion: We came to a conclusion in our study that p16 and Ki-67 immuno markers can be used in future studies as prognostic indicators. The combined study of p16 immunohistochemistry and HPV detection may play an important role in the clinical management.

Introduction

Oropharyngolaryngeal cancer cases remain as major global health issues since many years and these cancers are associated with significant morbidity and mortality rate. Globally about five lakh new oral and pharyngeal cancers are diagnosed every year with oral cancer being the sixth most common cancer in the world. The GLOBOCAN project has estimated that the global incidence rate is 4.0 cases per 1,00,000 population per year and the

global mortality rate is 1.9 deaths per 1,00,000 population per year. [1]

Cancer has a multifactorial etiology and arises by a complex process involving a series of genetic alterations which leads to cellular proliferation and differentiation. The sequence of epithelial alterations starts from hyperplasia, atypical hyperplasia, dysplasia, carcinoma in situ to invasive carcinoma. [2]

The immunohistochemical studies in order to determine the tumour-associated antigenic constituents, commonly referred to as "tumour markers" has received considerable attention. [3]

Common early events associated with the premalignant lesions of the oral mucosa, pharynx and larynx include inactivation of the tumour suppressor gene CDKN2A. The studies showed that CDKN2A gene is inactivated in approximately 70% of human cancers. Its codified protein p16INK4a is a cell-cycle inhibitor that acts in the pRb-p16INK4a tumor suppressive pathway. Both premalignant and malignant lesions have been linked to inactivation of the CDKN2A gene by homozygous deletion. [4]

Ki-67 is a nuclear protein which is associated with cellular proliferation and was originally identified by Gerdes *et al*. [5] Ki-67 expression is absent in cells showing an arrest in cell cycle and starts to be expressed in the S-phase, progressively increasing through G2 phase which reaches a plateau at mitotic phase. As Ki-67 reaction exclusively involves the proliferating cells, the immunostaining with antibodies to Ki-67 antigen is well established as a quick and efficient method for evaluating tumour growth fractions . [6]

The study of p16 (INK4a) expression and its mechanism of regulation can help to look for better therapeutic strategies that can improve the clinical course of HNSCC patients. The immunoexpression Ki-67 protein in all proliferating cells indicate the prognostic value of many cancers. Ki-67 protein is a potential therapeutic target in cancer, and strategies that inactivate Ki-67 protein are a promising anti-proliferative approach, with potential applicability in cancer treatment. [7]

Therefore, this study is taken up to assess the immunohistochemical expression of p16 and ki-67 in oropharyngolaryngeal tumorigenesis and its degree of expression from benign and premalignant disorder to malignancy, which is also important for prognostification.

Materials and Methods

This 2-year cross-sectional study (September'18-August'20) was performed on 62 histopathologically proven neoplastic lesions of OPL region which includes 6 benign lesions,12 dysplastic lesions and 44 SCCs. The sample for this study was obtained from the Department of Pathology, Hi Tech Medical College & Hospital, Bhubaneswar, Odisha, India. From the paraffin-embedded blocks, two sections of 3µm were taken on poly-L-lysine-coated glass slides for immunohistochemical staining of p16 and Ki-67 respectively. The immunostaining was carried out using avidin biotin peroxidase technique. The antigen retrieval was performed using citrate buffer in the microwave oven set at 540º for 30 minutes. The slides were then incubated in 3% hydrogen peroxide solution. Ready-to-use mouse IgG-1 anti p16 monoclonal antibody (PathnSitu P16-JCB, R071240UA) and anti Ki-67 monoclonal antibody (Dako Monoclonal Mouse Anti-Human Ki-67 Antigen,20070470) were used as primary antibodies and were incubated for 60 min, followed by super enhancer for 25 min. For secondary antibody application, slides were incubated with polymer-HRP (horseradish-peroxidase) reagent (Dako real envision, Peroxidase / DAB^{+}, 20080624) for 30 min. The bound antibody was visualized using 3, 3- diaminobenzidine (DAB) (DAB

tetrahydrochloride) solution as the chromogen and counterstaining was done with Mayer's hematoxylin. Granular brown staining of nuclei and cytoplasm of the epithelial cells was considered positive for p16. The intranuclear brown granular staining of tumor cells were considered positive for Ki-67. The positive control was taken from previously diagnosed case of SCC. The negative control consisted of the replacement of the primary antibody for 1% bovine serum albumin, diluted in phosphate saline solution (TRIS).

Guidelines for interpretation of p16 (Shah NG et al.[8]) -

0 - Negative (staining in <10% of cells)

1+ - weak staining (staining in 11% to 30% of cells)

2+ - moderate staining (staining in 31% to 50% of cells)

3+ - intense staining (staining in >50% of cells)

Guidelines for interpretation of Ki-67(Buch A et al.[9]) -

Ki-67 Labelling Index (LI) was calculated as: number of positive epithelial cells/total number of epithelial cells ×100.

Ki-67 LI was graded as:-

Grade 0 (LI = 0%)

Grade 1 (LI = 1%–25%)

Grade 2 (LI = 26%–50%)

Grade 3 (LI = 51%–75%)

Grade 4 (LI = >75%)

Statistical Analysis: was made considering the clinical, histopathological and immunohistochemical data. Then transformed to a master chart by using Microsoft excel sheet, which was then subjected to statistical analysis using chi square test by using SPSS, version 20. Analysis was done in the form of percentage and represented as tables and figures where necessary. P value of ≤ 0.05 is considered as statistically significant.

Results

In the present study, 9.7% cases were benign lesions, 19.3% cases were premalignant lesions and 71% cases were malignant lesions. Out of total 12 dysplasia cases, 7 (58.3%) cases showed mild dysplasia and 5 (41.7%) showed moderate dysplasia. Out of 44 cases of squamous cell carcinoma 36 cases (81.8%) were of well differentiated squamous cell carcinoma (WD SCC) and 8 cases (18.2%) were of moderately differentiated squamous cell carcinoma (MD SCC).

The lesions were more common in males (80.6%) than females (19.4%). The mean age was 51 years. Maximum number of patients were in the age group of 40-59 years. Majority of cases (58.1%) were from buccal mucosa followed by 27.4% cases from tongue, 9.7% cases from larynx and 4.8% cases from pharynx. Maximum number of cases had history of only tobacco chewing followed by smoking (40.3%). 16.1% of cases were non-addict out of which the female non-addict cases were more than males.

All the benign tumours were negative for p16 expression. Out of 12 cases of dysplastic lesions, 5 cases (41.7%) expressed p16. Out of 44 cases of SCC, 34 cases (77.3%) expressed p16. With progression of lesion from premalignancy to malignancy, a progressive increase in percentage of p16 positive tumor cells and increase in immunostaining intensity were observed. These associations of p16 staining was statistically significant (p value = 0.026).

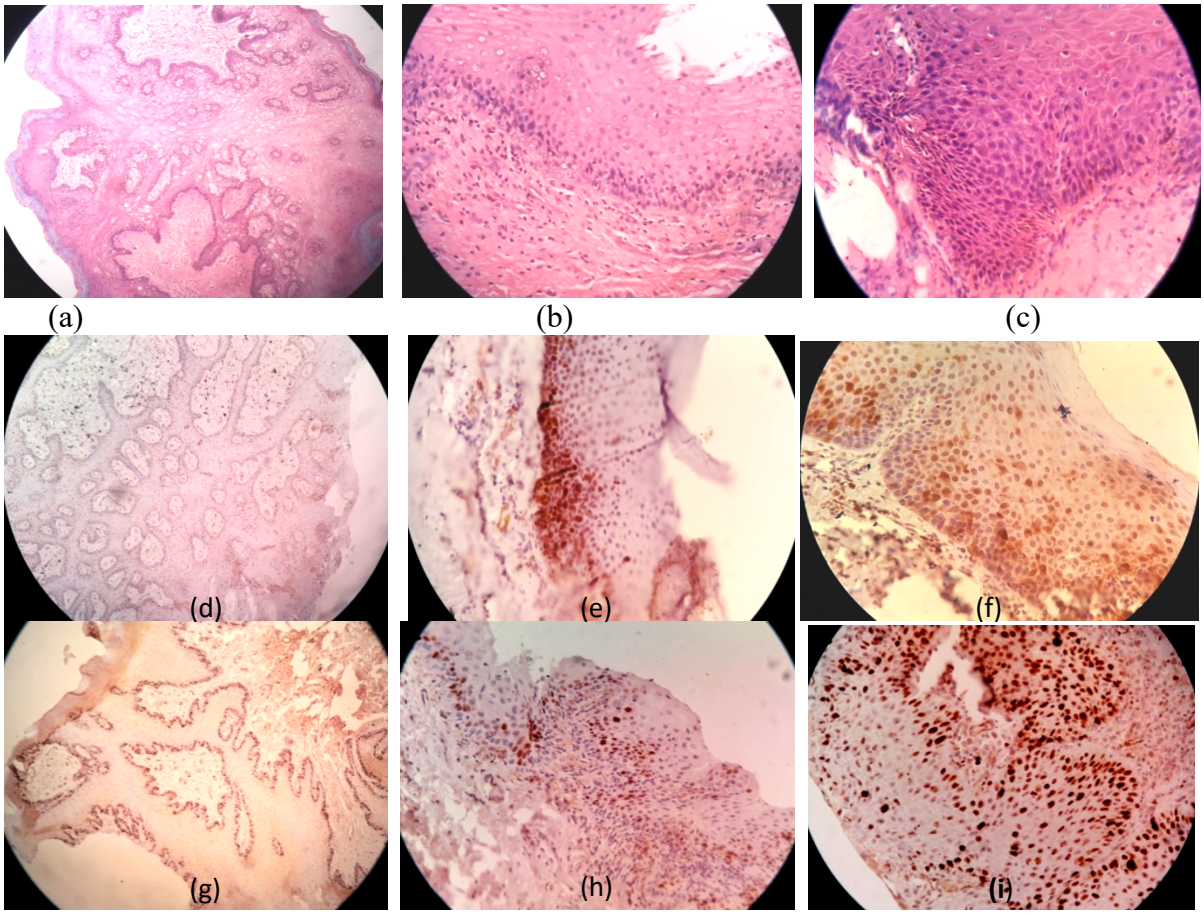
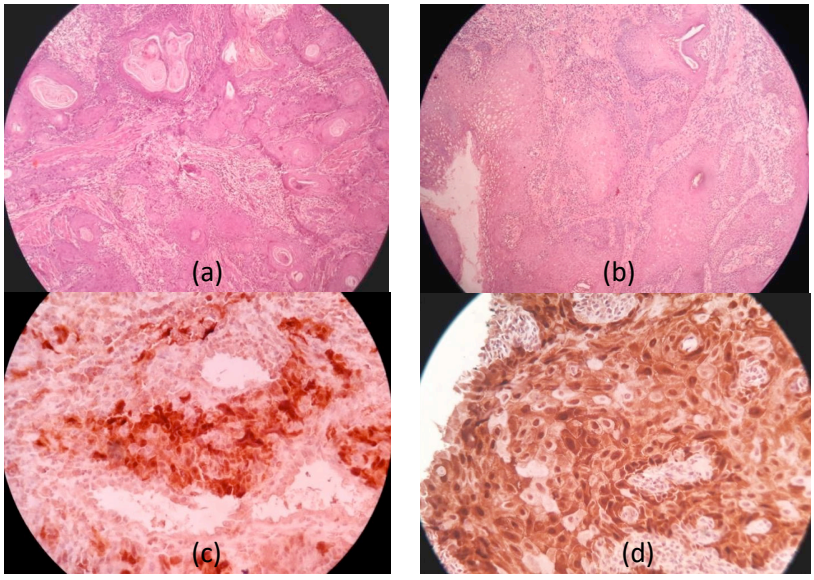


Figure 1: (a)squamous cell papilloma (H&E), (b)mild dysplasia (H&E), (c)moderate dysplasia (H&E), (d) p16 –ve sq cell papilloma, (e,f) p16 +ve mild & moderate dysplasia, (g,h,i)Ki-67 +ve sq cell papilloma, mild & moderate dysplasia

It was observed that 2 out of 6 benign lesions(33.3%) showed Ki-67 expression. All the 12 dysplastic lesions (100%) and all the 44 SCC (100%) expressed Ki-67. There was increase in Ki-67 labelling index with progression of lesion. The associations of Ki-67 LI was statistically highly significant (p value < 0.001).



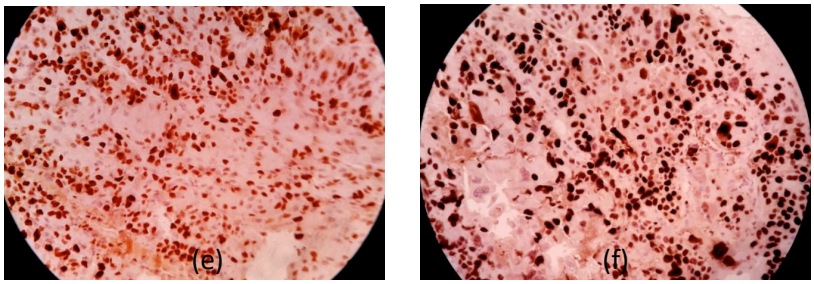


Figure 2: (a) WD SCC,H&E, (b) MD SCC,H&E, (c) p16 +ve, WD SCC, (d) p16 +ve, MD SCC, (e) Ki-67 +ve, WD SCC, (f) Ki-67 +ve, MD SCC

Discussion

This cross-sectional study included 62 cases of oropharyngolaryngeal neoplastic lesions. We took two immunomarkers, p16 & ki-67 for our study and we correlated these two markers with different histologic grades of the OPL tumours. The lesions were commonly located in buccal mucosa inside oral cavity. The histologic grading of SCC was done using Modified Broders malignancy grading [Pindborg JJ et al. 19 97].

It was observed that none of the benign lesions was p16 positive which is similar to the observation by Gologan O et al [10]. But Kerge S et al [11] and Paschalis C et al [12] observed p16 expression in few benign lesions of OPL region. Our study showed a progressive increase in p16 expression with progression from mild dysplasia (28.5%) to moderate dysplasia(60%). Angerio F et al [13] and Gonzalez JCC et al [14] found the similar observation in their studies. Bradley KT et al [15] observed a decrease in p16 positive expression with increase in grade of dysplasia. In our study, the immunoexpression of p16 was gradually increased from well differentiated SCC cases (75%) to moderately differentiated SCC cases (87.5%). Angerio F et al [13], Lewis JS Jr et al[16], Ma C et al [17] and Ralli M et al [18] also observed that overexpression of p16 more likely to be detected with higher grade of SCC. In contrast, Yuen P et al [19] and Wayne S et al [20] found loss of p16 expression in higher grade of SCC.

The inactivation of p16 protein is an early event in the carcinogenesis process preceding the progression from premalignant to malignant lesion. Three major pathways of p16 inactivation have been described: promoter hypermethylation, point mutation and homozygous deletion. The inactivation of p16 protein leads to loss of p16 immunoexpression with increasing grade of dysplasia [21].

But in our study, there was overexpression of p16 with increasing grade of dysplasia. The overexpression of p16 may be due to role of human papilloma virus(HPV) in carcinogenesis process. Overexpression of p16 occurs due to blockage of key regulatory gene pRb by HPV oncoprotein E7. Rb gene normally suppresses p16 transcription and HPV E7 protein targets the active hypophosphorylated pRb [22] In the present study, there was a statistically significant correlation (p value=0.026) between p16 immunoexpression and increase in grade of tumours from benign and dysplastic lesion to SCC.

The Ki-67 immunoexpression in our study showed all cases of dysplasia and SCC were positive for Ki-67 and all benign lesions were negative for Ki-67 expression. The similar observations were also seen by Angerio F et al [13], Dragamoir LP et al [23], Takkem A et al [24]. Our study also showed an increase in Ki-67 expression with increasing histological grade of lesions (p value < 0.001). The expression of Ki-67 is mainly limited to parabasal layer 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. Its expression is found in all layers of dysplastic lesions and SCC as number of cells increase in a cell cycle. [25]

Conclusion

An attempt was made in this study to understand the correlation of p16 and Ki-67 expression with benign lesions, different histological grades of dysplastic lesions and SCC. Gradual increase in p16 and Ki-67 immuno staining intensity was observed with progression of lesion from mild to moderate dysplasia as well as from WD SCC to MD SCC. Thus, these markers can be used in future studies as prognostic indicators. The overexpression of p16 was described in many studies to be associated with HPV induced carcinogenesis. So the combined study of p16 immunohistochemistry and HPV detection may play an important role in the clinical management. We concluded that the immunohistochemical assessment of p16 and ki-67 along with histological grading will guide the clinicians to make appropriate choice for treatment protocols.

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