Original Article

Cell Proliferation Marker Ki-67, Its Prognostic and Predictive value in Squamous Epithelial Dysplasias and Squamous cell Carcinoma of Oropharynx

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ABSTRACT

Background and Aim: Antigen Ki-67 is a nuclear histone protein associated with cellular proliferation and expressed in certain phases of cell cycle (S, G1, G2, and M). Over expression seen in malignant tissues and associated with poor prognosis hence it is used as biomarker in neoplastic tissues.

Aim of Study : Study of expression of Ki-67 labelling index in various grades of dysplasias and squamous cell carcinomas.

Materials and Methods: The sample consisted of 100 formalin-fixed, paraffin-embedded specimens of oral pharyngeal squamous epithelial dysplasia (OPSED), oral squamous cell carcinomas (OPSCC)were conventionally stained with hematoxylin and eosin then immunohistochemically stained with Ki-67 monoclonal antibody.

Results: Expression of Ki-67 was restricted to the basal layers in the normal oral epithelium whereas Ki-67 positive cells were located in the basal, suprabasal and spinous layers, Ki-67 expression was increased in high-risk cases. Ki-67 positive cells in well-differentiated OPSCC were located mainly in the periphery of the tumor nests, in moderately-differentiated OPSCC were located in both peripheral and part of a center of the tumor nests whereas it was diffused in most of the Poorly-differentiated OPSCC. Statistical analysis indicated a significant difference be-tween the expression in OPSED and OPSCC .

Conclusion: This study has concluded that Ki-67 antigen could be used as a marker for the histological grading of and OPSCC (Oropharyngeal squamous cell carcinoma), Expression of Ki 67 increased according to the severity of squamous epithelial dysplasias and squamous cell carcinomas.

Keywords: Ki-67 antigen- Oropharyngeal squamous epithelial dysplasia (OPSED)- Oropharyngeal squamous cell carcinomas (OPSCC).

INTRODUCTION

Premalignant lesions of the oropharynx present as visibly abnormal areas of mucosa and may be a source of anxiety for the patient and the clinicians. Precancerous lesions should be biopsied to evaluate for dysplasia.[1] There are multiple genetic mutations that must occur from normal tissues to progress from dysplasias to carcinoma. Biopsy remains the gold standard for investigating dysplasias and squamous cell carcinoma.[2] Two common conditions of premalignant lesions were included in the current study; leukoplakia and erythroplakia.

leukoplakia is a white patch on the mucosa, which cannot be scraped off and cannot be attributed to any other disease process. 'Leuko' means white and 'plakia' means patch.[3] The rate of malignant transformation is higher in non-homogenous leu-koplakic lesions that demonstrate low grade to high grade dysplasia to malignant transformation, age >60 years with chronic smokers are more fre-quently associated with malignant transformation. The presence of High grade dysplasia is

the strongest indicator for malignant transformation with rates of 15-30%.[4, 5] Speckled leukoplakia, which is characterized by the presence of white and erythematous nodular patches were often associated with squamous dysplasias or invasive SCC. The term epithelial dysplasia is a histopathologic diagnosis rendered when cells with atypical morphology are detected within the epithelium and are graded by severity as dysplasias or carcinoma in situ. Epithelial dysplasia is histologically characterized by a stratified squamous epithelium with cellular atypia and loss of cell differentiation and stratification.[5] In epithelial tissues mutations, chromosomal damage, and loss of cellular control functions were manifested as the transition from normal histology to Dysplasias to carcinoma in situ and invasive squamous cell carcinoma. Correct diagnosis of premalignant lesions with high risk of malignant transformation may help to perform appropriate treatment with primary surgery, radiation, chemotherapy or both radiation and chemotherapy. Most of Squamous Cell Carcinoma progresses via a premalignant lesion called Intra epithelial lesions or dysplasias with a frequency up to 17.5%.[6]

Squamous cell carcinoma is the most common cancer of oropharynx. It is the most commonly seen in men than women with >40 years of age.[7] OPSCC may affect various sub sites of oropharynx (tongue, soft palate, tonsils, posterior and lateral pharyngeal wall) but most frequently the base of tongue.[8] The main risk factors associated with squamous cell carcinoma include tobacco/betel/areca nut use, alcohol consumption, infection with high-risk human papillomavirus (HPV) genotypes, and a diet low in fruits and vegetables.Various histopathological grading systems of OPSCC have been discussed in literature and Broder's grading has been popular for a long time. Broder's criteria to classify as well-differentiated squamous-cell carcinoma , moderately differentiated squamous-cell carcinoma and poorly differentiated squamous-cell carcinoma.[9]

INCIDENCE IN INDIA

Globally incidence is approxi-mately 3%, in India its prevalence is 0 to74%. Despite refinement of surgical techniques in the past few decades once invasive cancer is formed, the prognosis is poor with an average 5-year survival rate of 40% of affected patients.[10] Markers of proliferation could potentially be good candidates for improving the prognostic evalua-tion of premalignant lesion and OPSCC. Until now, a panel of mo-lecular markers has not been deter-mined that allows for a prognostic prediction of OPSCC. However, these new markers could be considered complementary to conventional prognostic evaluation. The control on cell proliferation biological process is thought to be lost in cancer, and many studies have reported that abnormal cell proliferation appears to be a precursor and may be a predictor of tu-mourogenesis.[11] The most common immunohistochemical markers used to study cell proliferation are proliferating cell nuclear antigen Ki-67 antigen.[12] The Ki-67 protein, which encodes two protein isoforms with molecular weights of 345 and 395 kDa, was identified by Scholzer and Gerdes in the early 80s.The Ki-67 protein has a half-life of ~1-1.5 hour, it is present during all active phases of the cell cycle (G1, S, G2, and M), but is absent in resting cells (G0 and early G1).[13, 14] In later phases of mitosis (during anaphase and telophase), a large decrease in Ki-67 levels occur. Human Ki-67 is a 3256-amino-acid protein recognized by a monoclonal antibody generated by injecting mice with nuclei isolated from Hodgkin lymphoma cells.[15] Major regions of the Ki-67 protein include; an N-terminal forkhead-associated (FHA) domain; a protein phosphatase (PP)1 binding domain; a large unstructured central region com-prising 16 tandem repeats of 122 resi-dues (in primates); and a C-terminal LR (leucine/arginine-rich) chromatin-binding domain.[5] The function of the repeats, which are encoded by a single huge exon, remains unclear. Ki-67 redistributes from the nucleolar cortex and dense fibrillar compartments during interphase, to the chromosome periphery during mitosis.[16] Ki-67 is highly abundant and the epitope rec-ognized by the Ki-67 monoclonal antibody (FKELF) is naturally amplified, being present on nine of the 16 Ki-67 repeats that comprise much of the polypeptide. These features make Ki-67 one of the best markers to assess cell proliferation and it is used as a reagent to aid in determining a patient prognosis for several tumour types, including breast cancer.[17,18] The prognostic value of Ki-67 protein has been investigated in a number of studies with its potential as a reliable marker having been shown in cancers of the breast, soft tissue, lung, prostate, cervix and central nervous system. It has been shown that blocking of Ki-67 protein either by microinjection of antibodies leads to inhibit the progression of the cell cycle. An increasing number of studies have suggested that Ki-67 protein may be an important factor in cancer grading and prognostic evaluation.[19] The aim of this study was to identify an association between Ki-67 protein expression and histological grades of Dysplasia to SCC. And the role of Ki-67 protein in the prognostic of different histological grades of epithelial dysplasia and squamous cell carcinomas.

MATERIALS AND METHODS

The sample consisted of 100 formalin fixed, paraffin-embedded blocks of which Low grade and High grade dysplasias (20, 25 respectively), oropharyngeal squamous cell carcinomas (55) were collected from March 2019 to June 2020 including all small biopsies from oropharyngeal region and large specimens includes Hemi glossectomies, segmental mandibulectomies and others. The age range of the 100 patients were from 20 to 75 years, the sex ratio

male and female was 2:1. Squamous dysplasias was subdivided into Low (20), High grade (25). Invasive SCC [55] were subdivided into well differentiated (20), moderately differentiated (20), poorly differentiated (15). squamous cell carcinoma (OPSCC) was subdivided into 20 well differentiated OPSCC, 20 moderately - differentiated OPSCC, 15 poorly-differentiated OPSCC. Paraffin sections of formalin-fixed tissues were used for both histological and immunohistochemical evaluation. Hematoxylin and eosin stained sections of 4 µ were used for routine histological examination.

Immunohistochemistry (IHC) Immunohistochemical (IHC) detection of Ki-67 was performed using polymer based IHC kit of DAB detection system. For IHC staining the sections were cut at approximately 4 µm thick-ness using rotatory manual microtome. For IHC, sections were placed on precoated slides and incubated for 1 hour at 60°C in an incubator. For antigen retrieval, the sections were placed in a 1mm citrate buffer (pH 6) and microwave was used with cycles of high, medium high, low and very low each lasting for 5 min and then cooled to room temperature. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min followed by washing in 0.05mm Trisbuffered saline (TBS) at pH 7.4. The sections were incubated with precisely diluted mouse monoclonal antibodies against Ki-67 (MIB-1 prediluted Dako, Japan) as primary antibodies for 1 h at 37°C. Subsequently, after washing in TBS, the sections were incubated with a secondary antibody conjugated with peroxidase-labelled dextran polymers (Envision + Dual Link/HRP System, Dako) for 30 min at room temperature. After rinsing with TBS, they were treated with 0.5 mg/ml3, 3'-diaminobenzidine solution containing 0.001% hydrogen peroxide to visualize reaction products, and counterstained with Harris hematoxylin for 3 min.

Assessment of Expression of Ki-67

Nuclear staining either as coarse or fine granular dots considered posi-tive. The intensity of staining and the number or percentage of positive cells were assessed.

Grading of Ki-67 expression were evaluated using scores from 1 to 3.

1. 3+ - High proliferation - >50% +ve cells
2. 2+ - Moderate proliferation - 30-50% +ve cells
3. 1+ - Low Prroliferation - 10-30%+ve cells

MIB-1 labelling index was calculated by number of positive cells per 100 squamous epithelial cells in different areas under 100x magnification and the mean was calculated. Positive nuclei were expressed as the percentage of total nuclei counted.

Labelling index = (Number of cells showing +ve staining / total number of cells) x 100

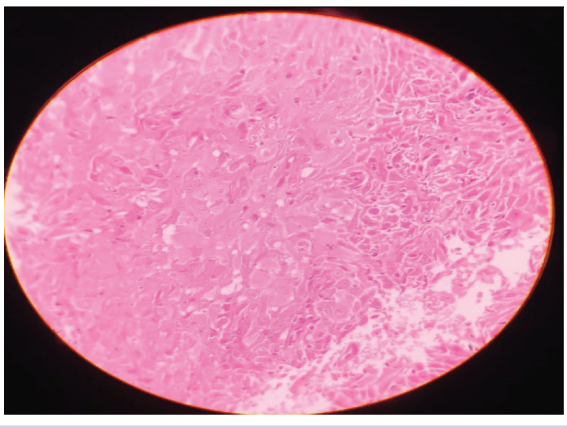


Figure 1: Severe squamous epithelial dysplasia

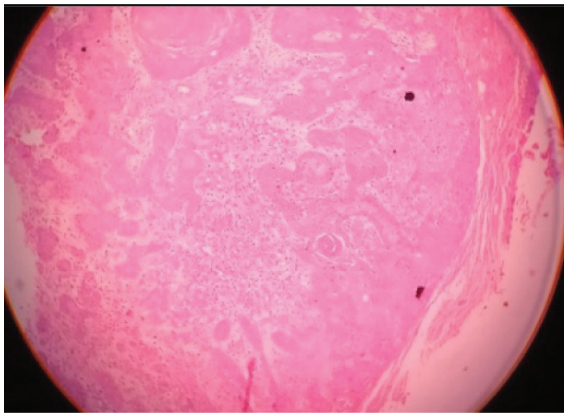


Figure 2: Well differentiated squamous cell carcinoma

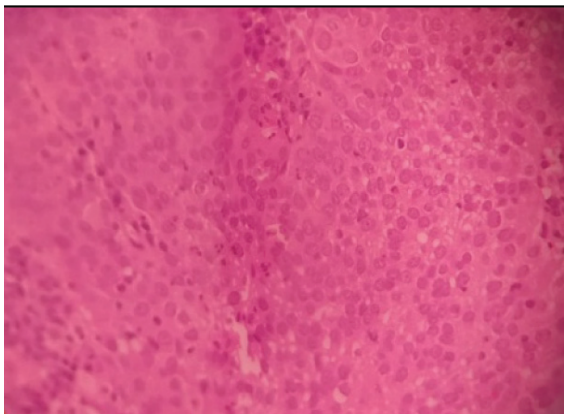


Figure 3: Poorly differentiated squamous cell carcinoma

STATISTICAL ANALYSIS

All immunohistochemical slides were examined for positive staining by light microscopy. The mean percentage of positively stained cells were estimated by counting 300 cells per area from at least five varied areas representative of the lesion's histology. The nuclear expression of Ki-67 was counted according to epithelial layers as the basal layer, parabasal layer, and suprabasal layer using a microscope at × 100.

Statistical comparison of Ki-67 protein expression in Oropharyngeal squamous Dysplasia group and Oropharyngeal squamous cell carcinomas group revealed a Significant difference, P=0.001.

Data are expressed as the means of percentage of positive cells 95% confidence intervals. The expression of the Ki-67 protein in the various lesions were analyzed for significance using oneway ANOVA test to compare the mean Ki-67 protein among various study groups, a P-value of <0.05 indicated a significant difference.

Statistical analysis indicates significant differences between the expression in Low grade, High grade dysplasias and Invasive carcinomas and normal Oropharyngeal epithelium, showed 'P' value of 0.001, the difference was not statistically significant between normal Oropharyngeal epithelium and Low grade dysplasia group (P > 0.05), we conclude that the rate of malignant transformation is higher in lesions that demonstrate High grade dysplasia.

Statistical analysis indicated significant differences between the expression in well-differentiated OPSCC group, moderately differentiated OPSCC group and poorly differentiated OPSCC group 'P' value of 0.001. We conclude from that the expression of Ki-67 increased progressively according to the grades of OPSCC.

RESULTS

- Oropharyngeal-Squamous Epithelial Dysplasias (n=45)
- Low grade (n=20) - The ki-67 expression was detected in basal layers.
- High grade (n=25) - The ki-67 expression was detected in all layers.
- OPSCC (n=55)
- Well differentiated(n=20)- The ki-67 expression was detected in all cases and was located at the periphery of the tumour nests than the centre.
- Moderately differentiated (n=20) -The ki-67 expression was detected in all cases and was located at the periphery of the tumour nests and part of the centre.
- Poorly differentiated (n=15)- The ki-67 expres-sion was detected in all cases of moderately differentiated SCC and was located at the periphery of the tumour nests and most of the centre.

DISCUSSION

The transition of the normal Oropharyngeal epithelium to Squamous dysplasias and to malignancy is featured by increased cell proliferation.[20] The control on cell proliferation is thought to be impeded in cancer, and many studies have reported that abnormal cell proliferation appears to be a precursor as well as a predictor of tumourogenesis.

As epithelial lesions is characterized by the number of cells and tissue alterations at a molecular and genetic level, there is an alteration of the cellular maturation in the epithelium leading increasing in the proliferative activity of the suprabasal layer.[21]

Proliferative activity in oro-pharangeal intraepithelial lesions and squamous cell carcinomas can be determined by immunohistochemistry using antibodies reactive against various proliferating cellular antigens. Cell proliferation markers play an important role in the biological behavior of neoplasms .

Discovery of various proliferation markers has enabled the detection of the hyperactive state of the epithelium and has been suggested to be of prognostic significance .[22] The monoclonal antibody Ki-67 was first described in 1983 and suggested that it might be used as a marker for proliferating cells.

A number of diagnostic applications for Ki-67 protein have been described, where Ki-67 was significantly more highly expressed in malignant than in normal tissues.[23] The significance of Ki-67 protein as a prognostic marker has been widely studied, Ki-67 represents an additional predictor of survival in breast cancer 24 cervical and uterine cancer, non Hodgkin's lymphoma and large bowel cancer and the expression of Ki67 can be used as a prognostic biomarker in Colorectal cancer (CRC).

Furthermore, Ki-67 is considered to be one of the best predictors of survival and recurrence. Increasing evidence indicates that Ki-67 protein as a prognostic marker. It, therefore, merits further development and study. In this study, the expression of the cell cycle-associated protein Ki-67 was examined by immunohisto-chemistry and normal mucosal epithelium as a control. And this article reviews the significance of Ki-67 protein as markers in diagnosis and prognostic assessment of severity of oropharangeal intraepithelial lesions and histological grades of OPSCC.

Table 1: Showing Incidence of Dysplasias and SCC

Type of Dysplasia	Number of cases	Percentage
Low grade Dysplasia	20	20%
High grade Dysplasia	25	25%
SCC	55	55%
TOTAL	100	100

Table 2: Showing Ki-67 positive cells in Dysplasia and SCC

S.No	Expression	Number of cases
1	Lower 1/3rd	20
2	Middle and upper 3rd	25
3	Lower, middle, upper 3rd and basement mem-brane invasion	55

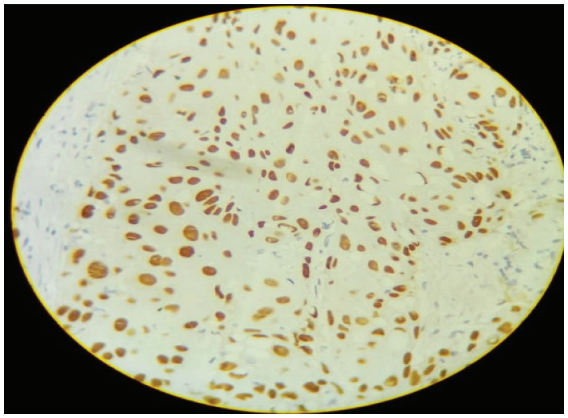


Figure 4: Severe squamous dysplasia

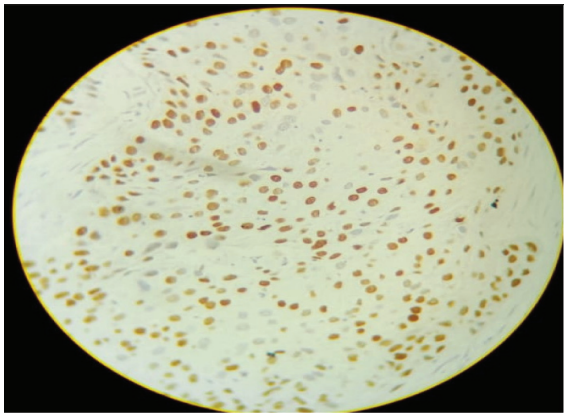


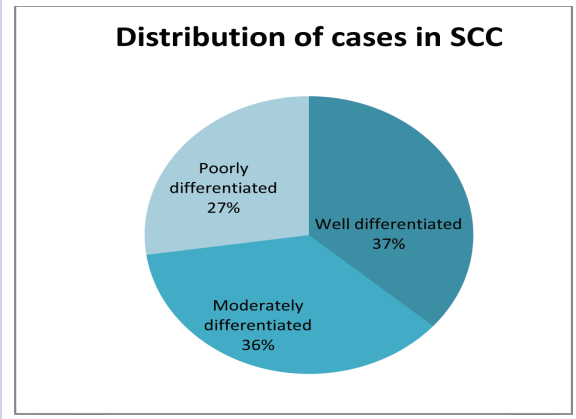
Figure 5: Moderately differentiated squamous cell carcinoma

Table 3: Showing Ki-67 scoring in LSIL, HSIL and Invasive SCC

	Ki-67 Score	Number of cases	Percentage
Low Grade	0	8	40
	1	12	60
High Grade	2	10	40
	3	15	60
Invasive SCC	2	10	18
	3	45	81
TOTAL		100	

Table 4: Distribution of Ki-67 cells

Sl. No.		Squamous epithelial cell layer	Low Grade Dysplasia	High Grade Dysplasia	SCC
1	With No to minimum expression		8	0	0
2	With Ex-pression	Lower 1/3rd	12	0	0
		Lower middle 1/3rd	0	10	0
		All layers	0	15	0
		All layers with inva-sion	0	10	45



In normal oropharyngeal epithelium, the proliferating cells were restricted mainly in the basal layer of the epithelium this was in accordance with pre-vious studies. In our study, we found to that expression of the Ki-67 protein in the located at the basal layer and, parabasal, in some cases spinous layers and its expression increased with

Table 5: Comparative study of Ki-67 expression in Percentage

Author Name	Low Grade Dys-plasia	High Grade Dys-plasia	SCC
Wats & bose et al (2009)	39.1	98	100
Caval cante et al (2012)	32	74	74
Kanjana Kanthiya et al (2013)	22.6	75.4	90.6
Our Study	60	100	100

the severity of disease. In Low grade dysplasia, the maximum expression of Ki-67 was located at the basal and parabasal layers of the epithelium, which showed the least expression. As there was no statistically significant difference between the Low grade dysplasia and the normal oropharyngeal epithelium (NOE), it can be concluded that it is very difficult to pre-dict the prognosis of Low grade lesions, as it is more or less, not very aggressive and has proliferative activity similar to normal epithelium.

In High grade dysplasia, The nuclear Ki-67 positivity was found in the basal, parabasal and some of the spinous layers of the epithelium. There was the statistically significant difference between the normal oropharyngeal epithelium, Low grade dysplasia and High grade dysplasia and the nuclear Ki-67 positivity was found in the basal, parabasal and most of spinous layers of the epithelium it can be concluded that it is the rate of malignant transformation is higher. In future, the Ki-67 protein may serve as prognostic tools in the detection of malignant transformation in oropharyngeal lesions. And we found to that cell proliferation in OPSCC increasing according to histological grades by an antibody for Ki-67 protein this was in accordance with previous studies. In well-differentiated OPSCC the nuclear Ki-67 positivity was found in the pe-ripheral area of tumour islands.

This suggests that less differentiated cells are located in the peripheral layer and the central cells are highly differentiated with the ability of keratinization, thus, no expression of Ki-67 was observed in the central cells of the tumour island. In moderately differentiated OPSCC, the nuclear Ki-67 positivity was found in the peripheral area and part of the center area of tumour islands. The overall staining of Ki-67 in moderately differentiated OPSCC was more quantitatively than welldifferentiated OPSCC.

In poorly differentiated OPSCC the nuclear Ki-67 positivity was found in the peripheral area and most of the central area of tumour islands the staining of Ki-67 was diffuse, it can be concluded that the cells were less differentiated and in more proliferating phase. The high expression of the Ki-67 protein in high grade Squamous dysplastic tissues may play an important role in the development of oropharyngeal squamous cell carcinoma Ki-67 protein increase in expression with decreasing tissue differentiation of high grade dysplasia and OPSCC. The difference between Squamous dysplasias and OPSCC was not significant, thereby signifying the fact that Dysplastic epithelium holds a high potential for malignant transformation. Because of expression Ki-67 protein in all proliferating cells and the prognostic value of the Ki-67 marker in 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.

CONCLUSION

In conclusion, the cell proliferation in Squamous dysplasias and oropharyn-geal squamous cell carcinomas can be determined by its growth rate using anti-Ki-67 monoclonal antibody. The nuclear protein Ki-67 is an established prognostic and predictive marker for the assessment of biopsies from patients with Squamous dysplasias and oropharyngeal squamous cell carci-noma. The Ki-67 expression is significantly higher in tissues with moderately differentiated or poorly differentiated squamous cell carcinoma and High grade dysplasia and provides an objective criterion for determining the severity of dysplasia and histological grading of OPSCC.

CONFLICT OF INTEREST:

The authors declared no conflict of interest.

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