Research Article

Comparison of Ki-67 Expression in Leukoplakia and Squamous Cell Carcinoma-A Retrospective Study

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ABSTRACT:

Background: Oral malignancy is the most common malignancy worldwide. In India, approximately 77000 new cases are diagnosed and 52000 deaths are reported annually.

Absolutely, understanding the molecular pathogenesis of conditions like oral leukoplakia can indeed be a game-changer in terms of early diagnosis and prognosis. Cell proliferation, which is tightly regulated in healthy tissues, can go haywire in cancer, leading to uncontrolled growth and tumor formation. By studying the molecular mechanisms underlying this dysregulation, researchers can identify potential biomarkers for early detection and develop targeted therapies to intervene in the progression of the disease. In the present study Ki-67 expression was compared between lesions of leukoplakia and Oral squamous cell carcinoma. Grades of epithelial dysplasia and Oral squamous cell carcinoma were also evaluated.

Materials & Methods: This retrospective study conducted at our institution, represents a significant effort to understand the proliferative activity in oral lesions. With a total sample size of 135, including 72 oral leukoplakia samples, 50 oral squamous cell carcinoma samples, and 13 normal mucosal samples, it provides a comprehensive view of Ki-67 expression in different oral conditions.

Results: The finding of over expression of Ki-67 with increasing grades of Oral Epithelial Dysplasia and in Oral Squamous Cell Carcinoma compared to normal mucosa was significant (p-value<0.05) and aligns with the known biology of these conditions.

Conclusion: The conclusion drawn from our study suggests that Ki-67 expression could indeed serve as a reliable marker for predicting the future outcome of Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma.

KEYWORDS: Ki-67, epithelial dysplasia, carcinoma, leukoplakia

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Email Address: drpankajr1982@gmail.com,
Submitted: 07.01.2024, **Accepted:** 03.05.2024, **Published:** 13.06.2024

INTRODUCTION:

Indeed, head and neck cancers represent a significant public health challenge worldwide. These cancers can affect various areas of the head and neck, including the oral cavity, pharynx, larynx, nasal cavity, paranasal sinuses, and salivary glands. The rise in the number of new cases each year adds to the burden faced by individuals, families, healthcare systems, and society as a whole[1,2].

The burden of oral cancer in nations undergoing economic transition, such as India, is indeed a significant public health challenge. India, in particular, faces a substantial number of new cases and

Access this article online

Quick Response Code:

Website: www.pjsr.org

DOI:

How to cite this article: Rathod P, Singh A, Nagi R. Comparison of Ki-67 Expression in Leukoplakia and Squamous Cell Carcinoma-A Retrospective Study. PJSR:2024;17(1):9-16.

deaths from oral cancer each year, highlighting the urgent need for effective prevention, early detection, and treatment strategies. Approximately 77,000 new cases and 52,000 deaths annually are staggering and underscore the magnitude of the problem in India[3,4].

Absolutely, tobacco use, alcohol consumption, and exposure to environmental carcinogens are indeed major risk factors associated with oral cancers [5,6,7,8].

Oral pathologists play a crucial role in the early detection and prevention of oral cancers. Their unique position allows them to identify potentially cancerous lesions in the symptomless phase, often before patients are even aware of any abnormalities. This early detection is key because oral cancers, when diagnosed at an early stage, are more treatable and have a better prognosis. However, many patients may not fully understand the seriousness of oral cancers or may delay seeking medical advice due to various reasons, including lack of awareness, fear, or stigma associated with cancer diagnosis. This delay in seeking medical attention can allow the disease to progress rapidly, making treatment more challenging and reducing the chances of a successful outcome. To address this issue, oral pathologists can play a proactive role in raising awareness about the importance of regular oral screenings and early detection of oral cancers. By educating their patients about the risk factors associated with oral cancers, the signs and symptoms to watch out for, and the benefits of early intervention, oral pathologists can empower individuals to take charge of their oral health and seek timely medical advice if needed. Oral leukoplakia (OL) is one of the most common precancerous lesions[9,10].

Leukoplakia is defined as "A white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer"[11].

Absolutely, advancements in molecular biology have significantly contributed to our understanding of the molecular pathogenesis of oral cancer and have led to the development of newer diagnostic and treatment techniques. Molecular studies have unraveled the intricate mechanisms underlying the development and progression of oral cancer, shedding light on molecular pathways, genetic mutations, and aberrant cellular signaling that drive carcinogenesis.

One key aspect of this progress is the identification and characterization of tumor markers. Tumor markers are substances that can be detected in the blood, urine, tissue samples, or other bodily fluids of patients with cancer. These markers are often proteins or genetic materials that are either produced by cancer cells themselves or released by the body in response to the presence of cancer.

The detection and analysis of tumor markers hold immense potential in the diagnosis, prognosis, and treatment of oral cancer[12,13].

It is hypothesized that Cancer is characterized by uncontrolled cell proliferation, which means that cells divide and multiply at an abnormal rate, leading to the formation of tumors[14].

Ki-67 is indeed an excellent marker for assessing the growth fraction or proliferative activity of a given cell population. Its presence throughout the active phases of the cell cycle—G1, S, G2, and mitosis—makes it a reliable indicator of cells that are actively dividing and proliferating.

Since Ki-67 is absent in resting cells (G0 phase), it provides a dynamic snapshot of the proportion of cells within a population that are actively cycling at any given time. This information is crucial for understanding the proliferative status of tissues, assessing the aggressiveness of tumors, and predicting their behavior.

In research and clinical settings, immunohistochemical staining for Ki-67 is commonly used to quantify the proliferative index or labeling index of tumor cells. By measuring the percentage of cells expressing Ki-67 in a tissue sample, researchers can estimate the rate of cell proliferation and correlate it with various clinical parameters, such as tumor grade, stage, and prognosis[15].

We Compared Ki-67 expression levels between lesions of OL, oral squamous cell carcinoma (OSCC), and normal oral mucosa (OM). Utilizing immunohistochemistry to assess Ki-67 expression allows for a quantitative analysis of the proliferative activity within these tissue samples.

MATERIALS & METHODS

This study was done in our institute. The study included 135 samples in total. Out of these, 72 were known OL samples, while 50 were known OSCC. 13 normal mucosal samples were taken. The normal samples were obtained from patients undergoing third molar extractions, they were informed and their approval was sought. Institutional ethical clearance for the study was obtained. The formalin fixed paraffin embedded (FFPE) tissue blocks were obtained from the department of Oral Pathology.

The blocks were sectioned at 4 micron thickness. 2 sets of slides were made, 1 for Hematoxylin & Eosin (H&E) and the other for Ki-67. Gelatin chrome coated slides were used for proper adherence of tissue sections. The slides were stained

Table 1: Gender distribution of the samples.

Gender	Oral Leukoplakia		Oral Squamous cell carcinoma	
	No. of patient S (n)	Percentage (%)	No. of patient S (n)	Percentag e (%)
Male	65	90.27	45	90
Female	07	9.72	05	10
n	72	100	50	100

Table 2: Distribution of cases according to dysplasia in OL

Group	Histopathological grading	No. of samples (n)	Percentage (%)
I	Mild epithelial dysplasi	30	41.66
II	Moderate epithelial dysplasia	23	31.94
III	Severe epithelial dysplasia	19	26.38

with H&E. This was followed by immunohisto-chemical evaluation (Ki-67). Antigen retrieval was done by placing sections in pressure cooker containing 1 mM EDTA buffer (pH 8.0) heated to 130C for 2.5 min and then cooled to room temperature. The endogenous activity was blocked by using 0.6% methanol Hydrogen peroxide for 15-20 minutes. This was followed by rinsing with distilled water and tris buffer wash (pH 7.2-7.6). The slides were inserted in a petridish containing citrate buffer and placed in a microwave oven at 700 watts for 10 minutes. The petridish was allowed to cool to room temperature for 20 minutes and then the slides were taken out and washed with tris buffer. Excess buffer was washed and finally sections were covered with optimally diluted primary antibody and incubated at room temperature for 1 hour. After washing in wash buffer, excess was removed and this was followed by treatment with biotinylated rabbit anti mouse IgG and incubated for 30 minutes. Again the slides were washed with buffer. Enough drops of streptavidin peroxidase were applied to cover the slides and were incubated for 45 minutes. Freshly prepared substrate chromogen solution was applied to cover the sections and incubated at room temperature for 6-7 minutes. Finally, the slides were counterstained with Harri's hematoxylin. The sections were dehydrated, cleared and mounted in DPX (Distrene Debutyl-Pthalate Xylene)[16].

INTERPRETATION:

Histopathological diagnosis was confirmed in H&E stained sections. This was followed by analyzing immunohistochemically stained sections for Ki-67 at different magnifications. Random 10 fields were used for the quantitative assessment of Ki-67 positive cells at high power. Intranuclear light brown granular staining confirmed ki-67 positivity. Expression of Ki-67 less than 10% were considered negative.

RESULTS:

This study carried out in our institute was planned to correlate the ki-67 expression with histopathological grades of OSCC and OL. In a total of 72 OL, 65 were males and only 7 were females and out of 50 OSCC, 45 were males and 5 were females [Table1]. Out of 72 OL samples, 30 were mild dysplasia (Group I), 23 moderate dysplasia (Group II) and 19 severe dysplasia (Group III) [Table-2]. Similarly, out of 50 OSCC samples, 30 were well differentiated (Group IV), 15 were moderately differentiated and 4 were poorly differentiated. Since poorly differentiated were very few they were clubbed with moderately differentiated and comprised of Group V (n=20). 13 samples from normal patients formed Group VI [Table 3]. The evaluation of the slides was done by scanning the immunohistochemically stained slides and counted in five different histological fields using a magnification of 40X. An eyepiece grid was used to prevent the overlapping of fields. Comparisons were made between Group I, II and III each with Group VI. Similarly, Group IV and V were compared with Group VI. Histological grades of dysplasia in OL were also compared with histological grades of OSCC. The indices were calculated as the percentage of positively

Table 3: Distribution of cases according to histopathological grading in OSCC.

Group	Histopathological grading	No. of samples (n)	Percentage (%)
I	Well differentiated squamous cell carcinoma	30	60
II	Moderately & Poorly differentiated squamous cell carcinoma	20	40

Table 4: Comparison of Grades of OL with normal mucosa.

Control group n=13 [Group VI]	Mild epithelial dysplasia n=30 [Group I]	Moderate epithelial dysplasia n=23 [Group II]	Severe epithelial dysplasia n= 19 [Group III]	*f*-value	*p*-value
2.35 ±15.62	40.37 ±6.42	43.14 ±4.04	47.67 ±3.31	29.42	0.001

Table 5: Comparison of Grades of OSCC with normal mucosa.

Control group n=13 [Group VI]	Well Differentiated n=30 [Group IV]	Moderately & Poorly Differentiated n=20 [Group V]	*f*-value	*p*-value
2.35 ±15.62	75.74 ±19.62	92.32 ±15.67	48.71	0.001

stained cells among the total number of cells. The results were tabulated and subjected to statistical analysis including Student's t-test, Chi-square test and Mann-Whitney U-test. The comparisons showed overexpression with increasing grades of Oral Epithelial Dysplasia (OED) and in cases of Oral Squamous Cell Carcinoma (OSCC). Ki-67 overexpression was seen increasing from Grade I to Grade III [Figure 1, 2 & 3]. *p*-value was significant [Table 4]. Similarly, comparisons amongst Group IV and Group V [Figure 4] too showed increase in the mean value of Ki-67 positive cells. *p*-value was significant [Table 5]. Finally a comparison was done between OED and OSCC which showed increased value in OSCC. These results were significant (*p*-value<0.05).

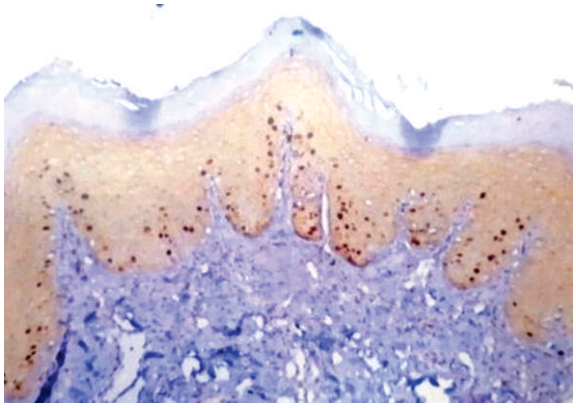

Figure 1: Mild epithelial dysplasia IHC (OL).

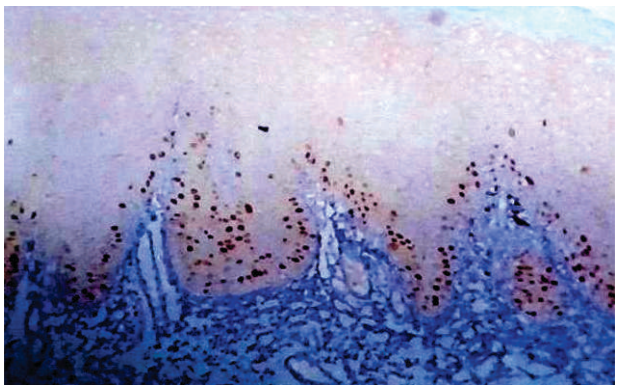

Figure 2: Moderate epithelial dysplasia IHC (OL).

DISCUSSION:

The ability to reliably predict cancer outcomes can significantly impact the management and biological behavior of lesions, aiming to achieve optimal results in terms of loco-regional control, overall survival, and quality of life. It is well-established that oral lesions (OLs) exhibiting a

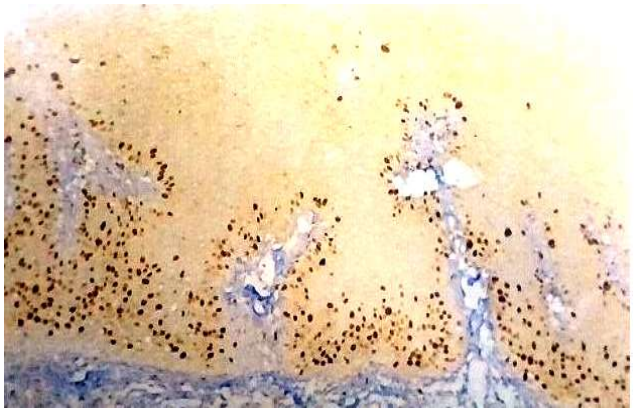

Figure 3: Severe epithelial dysplasia IHC (OL).

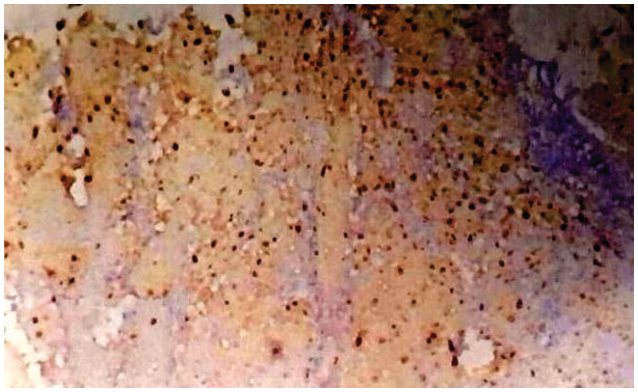

Figure 4: Poorly Differentiated squamous cell carcinoma IHC.

histologically moderate to severe degree of epithelial dysplasia have a higher propensity for malignant transformation[17,18]. H& E staining is used to routinely diagnose dysplasias. However, the assessment of epithelial changes in the oral mucosa has recognized limitations, representing a significant constraint in predicting the behavior of suspicious lesions. These limitations arise from several factors such as Subjectivity in Histopathological Evaluation, Sampling Errors, Biological Diversity of Lesions, Lack of Molecular Insights.

The study of cellular proliferation markers such as PCNA and Ki-67 continues to be a vital aspect of understanding disease pathology, particularly in oncology. Their role in the diagnostic, prognostic, and therapeutic landscape makes them indispensable tools in modern medicine. Ongoing research and technological advancements promise to further refine their utility and application in clinical practice[19].

Epithelial dysplasia refers to the presence of abnormal cells within the epithelial tissue, indicative of an early step toward potential malignant transformation. This condition is marked by various cellular and tissue alterations, including disrupted cell maturation and increased suprabasal proliferative activity. Monitoring these changes in potentially malignant disorders (PMDs) can provide crucial insights into the risk of malignant transformation, particularly in tissues like the oral mucosa, cervix, and other epithelial surfaces. Epithelial dysplasia, marked by alterations in cell maturation and increased proliferative activity, is a critical factor in the progression of PMDs toward malignancy. Proliferative markers like PCNA and Ki-67 provide valuable information about the biological behavior of dysplastic lesions, enabling early detection and intervention, ultimately improving patient outcomes[20].

Cell proliferation markers are vital tools in understanding the dynamics of cell growth, especially in the context of diseases like cancer. These markers can be broadly categorized into three types: growth fraction markers, markers of specific cell cycle phases, and cell cycle time markers.

Categories of Cell Proliferation Markers:

1. **Growth Fraction Markers**:
   - These markers indicate the proportion of cells actively engaged in the cell cycle (G1, S, G2, and M phases) as opposed to resting (G0 phase).
   - **Ki-67**:
     - Ki-67 is a well-established growth fraction marker, present in all active phases of the cell cycle (G1, S, G2, and M) but absent in the G0 phase.
     - The intensity of Ki-67 staining and the number of positive cells is generally lower compared to PCNA. This is because Ki-67 is rapidly degraded after mitosis (M phase), whereas PCNA accumulates in cells.
     - The monoclonal antibody for Ki-67 detects a nuclear antigen associated with a nuclear non-histone protein, making Ki-67 a reliable indicator of cellular proliferation.
2. **Markers of Specific Cell Cycle Phases**:
   - These markers are used to identify cells in particular phases of the cell cycle, providing detailed information about cell cycle dynamics.
   - **Examples**:
     - **Cyclins**: Different cyclins are expressed at various phases of the cell cycle (e.g., Cyclin D for G1 phase, Cyclin A for S phase, Cyclin B for G2/M phases).
     - **Phosphorylated Histone H3**: This marker is specifically associated with cells in the late G2 and M phases.

3. **Cell Cycle Time Markers**:
    - These markers measure the duration of specific phases or the entire cell cycle, helping to understand the timing and rate of cell proliferation.
    - **Bromodeoxyuridine (BrdU)**:
        - BrdU is incorporated into newly synthesized DNA during the S phase, allowing researchers to measure the length of the S phase and overall cell cycle time.
        - Detection is usually performed through immunohistochemistry or flow cytometry.

Ki-67 vs. PCNA

- **Ki-67**:
    - Present in all active phases of the cell cycle (G1, S, G2, and M), but not in G0.
    - Rapidly degraded after the M phase, leading to lower staining intensity and fewer positive cells compared to PCNA.
    - Reacts with a nuclear non-histone protein, which is essential for reliable detection of proliferating cells.
    - Considered one of the most reliable markers for cellular proliferation due to its specific expression pattern.

This antigen is present in the nucleoli in G1followed by nucleoplasmic distribution in later cycle with intensity increasing in S and G2 and being maximum during mitosis[21,22].

Ki-67 was introduced as a potential marker for proliferating cells goes to Johannes Gerdes et al in 1983. The word Ki is derived from the University of Kiel, where Johannes Gerdes in the laboratory of Herald Stein generated antibody. It was the 67th well of a 96-well microtitre plate and hence it was designated as Ki-67. After cloning and sequencing the gene, new antibodies were generated and they were named after the division at (M)olecular (I)mmunology (B)orstel. Ki-67 gene is located on human chromosome 10 (10q25)[23].

We Examined 72 cases of OL and 50 cases of OSCC and 13 cases of normal oral mucosa. Out of these, 93% (n=126) were smokers. Affected males were 90.27% in OL and 90% in OSCC.

We used monoclonal antibody Mib-1 was used on paraffin embedded tissue sections to quantify the Ki-67 expression in the epithelium of OL and OSCC. We compared OED with OSCC. In the context of normal mucosal epithelium, the distribution and expression levels of Ki-67 are typically minimal and predominantly restricted to the basal and parabasal layers. This specific localization reflects the normal proliferative activity of these layers, which are responsible for generating new cells to replace those shed from the surface. The expression of Ki-67 tends to become more pronounced and extensive as the severity of dysplasia increases, progressing from mild to moderate to severe. This trend reflects the increasing proliferative activity and loss of normal cellular regulation associated with higher grades of dysplasia. We found out that, the positivity even extended in the spinous cell layer in severe and some moderate OED. In our study, we found out that the peripeheral cells of the tumor islands showed more positivity compared to the inner cells in OSCC.

Our results were in accordance with other studies[14,24,25]. Ki-67 positivity was more pronounced with increasing grades. The results were significant. OSCC compared with OED too showed higher positivity. Suwasini S et al found that Ki-67 overexpression is directly proportional to metastasis to head and neck lymph nodes[20]. Similar results were also seen in various other studies[26,27]. There have been contradictory results regarding prognosis of OSCC and Ki-67 overexpression. Xie S et al, found that over-expression of Ki-67 in OSCC relates to poor prognosis although suitable treatment timely provided could improve the prognosis[28].

Gonzalez-Moles MA found that ki-67 was over expressed in well differentiated OSCC when compared with poorly differentiated OSCC and hence Ki-67 may not be a reliable prognostic marker[29]. The exact reason may be unclear but this may be again due to inadequate sample size.

Ki-67 is a very important and reliable tool for diagnosis and prognosis and has various advantages like simplicity and interpretation of results. Pharmacological modulators of cell proliferation and differentiation have been postulated as the future of cancer preventive drugs. Some of these agents like dimethylfluoronithine and retinoids have already been used in clinical trials of OL. Thus the use of Ki-67 could be of great value in montoring the effects of these agents during the course of future chemoprevention trials.

CONCLUSION:

We conclude that Ki-67 is a reliable marker to predict future outcome of OED and OSCC. It can be used routinely to monitor its progression and treatment modalities.

Financial Support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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