Assessment of Ki–67 in Healthy, Premalignant, and Malignant Lesions of the Oral Mucosa and its Predictive Role in Disease Progression

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Abstract

Objective: To study Ki–67 expression in relation to proliferative status in leukoplakia with mild dysplasia, well-differentiated squamous cell carcinoma, and healthy oral mucosa.

Study design: Sections from 20 cases of leukoplakia with mild dysplasia and 20 cases of well-differentiated squamous cell carcinoma that were histologically confirmed were stained with MIB-1 antibody using an advanced polymer staining system. Quantification of the immunopositive cells was performed using an image analyzer. The Ki–67 labeling index was expressed as the ratio of the number of cells stained by MIB-1 to the total number of cells counted per section (minimum of 1000 cells in consecutive fields).

Results: The mean Ki–67 labeling index score was 8.47±1.586 in healthy oral mucosa, 25.5±3.01 in premalignancy, and 29.7±3.04 in squamous cell carcinoma.

Conclusion: The antibody MIB-1 represents a robust and easily applicable marker of cell proliferation whose expression correlates well with the disease progression from premalignancy to malignancy.

Keywords: Ki–67, labeling index, oral leukoplakia, oral squamous cell carcinoma, MIB-1, tumor progression

Introduction

Oral cancer is the sixth most common cancer in the world, accounting for approximately 4% of all cancers and 2% of all cancer deaths (1). Its etiology is complex and both endogenous and exogenous factors are of importance. Clearly, tobacco is one of the most predominant exogenous agents, with alcohol being another (2).

In recent years, more emphasis has been placed on cancer prevention programs and related studies (3). In these programs, success has relied on the development of efficient, early detection methods to identify patients at high risk for the development of cancer. Several clinical and etiological factors have been identified as markers of various malignancies for the detection of these high-risk groups. However, these factors are not efficient enough, so studies using biological markers for this purpose are emerging. In this regard, understanding the biological changes that occur during preneoplastic stages is of paramount importance. Of the premalignant lesions, oral leukoplakia has greatest relevance in the study of the biology of carcinogenesis because the oral cavity is easily accessible for clinical examination and also because it has a multi-stage carcinogenesis pathway applicable to the concept of field cancerization (4).

The oral carcinogenesis pathway can be broadly classified histopathologically into healthy, nondysplastic, dysplastic, carcinoma–in situ, and invasive carcinoma. Given that cancer is characterized by uncontrolled cell proliferation, markers of proliferation are relevant to studying neoplastic lesions (4).

Methods for assessing cellular proliferation in human tumors will provide pathologists and clinicians with an objective system of grading. This information regarding kinetic data will aid in patient care (5).

The most common marker used for the detection of cell proliferation is Ki–67 antigen. In tissue sections, the Ki–67 antigen is used to localize the Ki–67 protein. The Ki–67 antibody was named after its place of characterization in Kiel, Germany and
because the clone producing the antibody was grown in the 67th well of the tissue culture plate (6, 7). MIB-1 is a monoclonal antibody that has been prepared by the fusion of spleen cells of BALB/C mice immunized with recombinant parts of the Ki-67 antigen with cells of the mouse myeloma cell line X63Ag8.653. The MIB-1 monoclonal antibody has been proven to be a Ki-67-equivalent antigen with the advantage over the classical Ki-67 antibody of demonstrating good binding in frozen and formalin-fixed materials (8).

In the present study, the monoclonal antibody MIB-1 was used to assess cellular proliferation in healthy, premalignant, and malignant lesions of the oral mucosa and its predictive role in disease progression and also to determine its correlation with tobacco usage.

**Materials and Methods**

Paraffin-embedded oral tissues were obtained from the archives of the Department of Oral Pathology and Microbiology, Subharti Dental College, Meerut. A total of 60 samples were examined including 20 healthy nondysplastic controls, 20 leukoplakias with mild dysplasia, and 20 well-differentiated squamous cell carcinomas. Histologically proven oral premalignant and malignant lesions were selected for the study. A section of tonsil known to express high levels of Ki-67 was used as a positive control. A negative control of tonsil in which primary antibody was omitted was also included with each staining set.

**Ki-67 immunolocalization**

Histwaxed sections (3-μm thickness) were dewaxed in xylene and rehydrated in graded alcohols. Sections were then immersed in 0.01 M sodium citrate buffer. Immunoreactivity was enhanced by microwave heating at 800 W until the fluid boiled and then the microwave oven setting was reduced to 400 W for 15 min. Endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxide for 10 min. Sections were then washed in Tris-buffered saline (TBS) for 5 min and incubated with anti-human Ki-67 antigen clone MIB-1 monoclonal primary mouse RTU antibody (DAKO Cytomation, for 2 h. They were again washed in TBS for 5 min and then incubated with peroxidase-labeled polymer for 1 h. Sections were washed in three changes of TRIS for 5 min each and then covered with freshly prepared substrate chromogen solution for 10 min. After the final washing, the sections were counter-stained with hematoxylin, washed, dehydrated, cleared, and mounted in DPX. Negative controls were prepared by omitting the primary antibody.

**Quantification of Proliferative Index**

Ki-67 activity was quantified by selecting the most densely and evenly labeled areas in the sections and assessing the labeling index from the ratio of the number of cells stained by Ki-67 to the total number of cells counted per section [minimum of 1000 cells in two to four consecutive fields at high power (×40)]. All nuclei with diffuse or dot-like brown nuclear staining irrespective of staining intensity were rated as positive for Ki-67.

**Statistical Analysis**

Results from each group of tissues (healthy oral mucosa, leukoplakia with mild dysplasia, and well-differentiated squamous cell carcinoma) were pooled; the relationship between tissue groups was analyzed using the unpaired Student’s t-test. A P value of less than 0.05 was considered to be statistically significant.

**Results**

Positive Ki-67 staining was seen in all specimens of healthy oral mucosa (Group A), leukoplakia with mild dysplasia (Group B), and well-differentiated squamous cell carcinoma (Group C) and the results obtained were compared among the three groups (Figs. 1–3).

The mean Ki-67 labeling index score was 8.47±1.586 in healthy oral mucosa, 25.5±3.01 in premalignancy, and 29.7±3.04 in malignancy (Table I). Complete information about each patient’s tobacco consumption was available for 40 cases,
Fig. 1. (Normal epithelium) Stratified squamous epithelium showing nuclear staining in basal & parabasal layer (×10, IHC, MIB-1)

Fig. 2. (Leukoplakia with mild dysplasia) Stratified squamous epithelium showing nuclear stain in basal & parabasal layer and few cells from suprabasal layer (×10, IHC, MIB-1)

Fig. 3. (Squamous cell carcinoma) Tumor epithelial islands showing nuclear staining in the cells at the advancing front; except for the cells at the centre within the pearl are devoid of staining (×10, IHC, MIB-1)

Table 1. Comparison of proliferative activity of Ki-67 in different groups

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Groups</th>
<th>Ki-67 Labelling index scores± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A (Normal oral mucosa)</td>
<td>8.47±1.586</td>
</tr>
<tr>
<td>2</td>
<td>B (Premalignancy i.e Leukoplakia with mild dysplasia)</td>
<td>25.5±3.01</td>
</tr>
<tr>
<td>3</td>
<td>C (well-differentiated Squamous cell carcinoma)</td>
<td>29.7±3.04</td>
</tr>
</tbody>
</table>

Table 2. Comparison of proliferative activity of Ki-67 in premalignancy with habits & without habits

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>Number</th>
<th>Ki-67 Scores Mean± SD</th>
<th>'t' calculated</th>
<th>Degree of frequency 'df'</th>
<th>'t' tabulated (18, 0.05)=2.10</th>
<th>Probability value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>With habits</td>
<td>13</td>
<td>27.19±3.76</td>
<td>1.0707</td>
<td>18</td>
<td></td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>2</td>
<td>Without habits</td>
<td>7</td>
<td>25.27±3.86</td>
<td></td>
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Table 3. Comparison of proliferative activity of Ki-67 in malignancy with habits & without habits

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>Number</th>
<th>Ki-67 Scores Mean±SD</th>
<th>'t' calculated</th>
<th>Degree of frequency ‘df’</th>
<th>‘t’ tabulated (18, 0.05)</th>
<th>Probability value</th>
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<tbody>
<tr>
<td>1</td>
<td>With habits</td>
<td>14</td>
<td>28.17±3.16</td>
<td>0.7750</td>
<td>18</td>
<td>P&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Without habits</td>
<td>6</td>
<td>26.79±3.84</td>
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Table 4. Comparison of proliferative activity of Ki-67 in premalignancy with age

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>Number</th>
<th>Ki-67 Scores Mean±SD</th>
<th>‘t’ calculated</th>
<th>Degree of frequency ‘df’</th>
<th>‘t’ tabulated (18, 0.05)</th>
<th>Probability value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25-50 years</td>
<td>12</td>
<td>25.71±3.17</td>
<td>1.5231</td>
<td>18</td>
<td>P&gt;0.05</td>
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<tr>
<td>2</td>
<td>50-75 years</td>
<td>8</td>
<td>23.14±4.01</td>
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Table 5. Comparison of proliferative activity of Ki-67 in Malignancy with age

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>Number</th>
<th>Ki-67 Scores Mean±SD</th>
<th>‘t’ calculated</th>
<th>Degree of frequency ‘df’</th>
<th>‘t’ tabulated (18, 0.05)</th>
<th>Probability value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25-50 years</td>
<td>13</td>
<td>24.94±3.91</td>
<td>1.1284</td>
<td>18</td>
<td>P&gt;0.05</td>
<td></td>
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<tr>
<td>2</td>
<td>50-75 years</td>
<td>7</td>
<td>22.76±4.23</td>
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Table 6. Comparison of proliferative activity of Ki-67 in premalignancy with sex

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>Number</th>
<th>Ki-67 Scores Mean±SD</th>
<th>‘t’ calculated</th>
<th>Degree of frequency ‘df’</th>
<th>‘t’ tabulated (18, 0.05)</th>
<th>Probability value</th>
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<tbody>
<tr>
<td>1</td>
<td>Males</td>
<td>12</td>
<td>29.82±3.91</td>
<td>2.6918</td>
<td>18</td>
<td>P&lt;0.05</td>
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<tr>
<td>2</td>
<td>Females</td>
<td>8</td>
<td>25.16±3.61</td>
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Table 7. Comparison of proliferative activity of Ki-67 in Malignancy with sex

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>Number</th>
<th>Ki-67 Scores Mean±SD</th>
<th>‘t’ calculated</th>
<th>Degree of frequency ‘df’</th>
<th>‘t’ tabulated (18, 0.05)</th>
<th>Probability value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Males</td>
<td>14</td>
<td>31.68±3.98</td>
<td>2.9438</td>
<td>18</td>
<td>P&lt;0.05</td>
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<tr>
<td>2</td>
<td>Females</td>
<td>6</td>
<td>26.01±3.87</td>
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Discussion

Hyperproliferation is thought to be an early marker of disorderly growth. It is generally accepted that increased proliferation is associated with more advanced lesions and that the distribution of proliferating cells in tissue may reveal more about the regulatory mechanism that become dysfunctional during the multistep process of carcinogenesis (9).

The immunohistochemical markers used to study cell proliferation are PCNA, Ki–67, cyclin D, and CENP-F. The present study focuses on one of the proliferating markers, Ki–67, as stained by MIB–1, which is easy to interpret because there is low background staining and the nuclear stain is very intense. It is also believed to be a reliable marker of cell proliferation because this nonhistone nuclear protein can be expressed at all stages of the cell cycle except G0 (9, 10).

Ki–67 human nuclear antigen is expressed during the G1, S, G2, and M phases of the cell cycle, but is absent in the quiescent G0 phase. It is localized in the nucleoli in G1, with a nucleoplasmic distribution later in the cycle, increasing in intensity through S and G2, to reach a maximum at mitosis. In the S phase, the antigen was also detected homogenously in the karyoplasms and in the G2 phase, the staining within the karyoplasm existed as a mixed, finely granular, and speckled pattern with perinucleolar staining still present. Intense perichromosomal Ki–67 staining was noted during prophase and metaphase, in addition to karyoplasmic staining in prophase and cytoplasmic staining in metaphase. The intensity of staining then rapidly diminished during anaphase and telophase. The half-life of detectable Ki–67 antigen is very short, an hour or less due to the presence of numerous proline-glutamic acid-serine-threonine (PEST) motifs in the protein that facilitate rapid catabolism (11, 12).

In healthy epithelium in the present study, it remained confined to isolated cells/occasional cells adjacent to the basal lamina, i.e., in the basal layer and mostly in the parabasal layer with no positivity in the superficial layer, results that supported those of other studies. Therefore, it could be considered that transient amplifying cells derived from stem cells might be present in the parabasal layer of normal oral epithelium. The present study showed proliferative cells at abnormal sites within the epithelium, sometimes throughout the entire epithelial thickness, in premalignant lesions (13–17).

These findings supported those of Liu et al., who found that the basal and superficial layers showed the clearest differences between normal and abnormal tissues. Because of the higher number of cells and the relatively high proliferative activity in dysplastic leukoplasias, the superficial layer and the basal layer seem to be the most adequate tissue components to investigate the possible modulation of cell proliferation in precursor lesions (18–20).

Zidar et al. (1996) showed that in abnormal and atypical hyperplasias, there is a significant increase in the number of Ki–67–positive cells that appeared in the suprabasal portion of the epithelium, characterized by augmentation of immature basal-like cells that occupy the lower two-thirds and may extend up to the surface of the epithelium (21).

This dissertation evaluates the hypothesis that distributional alteration of proliferating cells and stem cells within epithelial dysplasia may be a useful index to estimate the development of epithelial precursor lesions by immunohistological examination.

In the present study, in squamous cell carcinomas, Ki–67 positivity was observed in peripheral tumor parts and in the cells in the periphery of tumor nests. When the tumor cells matured into squamous pearls, the positivity of Ki–67 disappeared. This staining pattern suggests that the tumor periphery is composed of highly primitive proliferative cells, which are the progenitor of the more differentiated cells in the central portion of the tumor, as suggested by Slootweg (22).

In light of these considerations, we hypothesize that different tumor types exhibit distinct cell cycle kinetics, which additionally depend on the degree of differentiation. Although the dynamics of mitotic cytokines are incompletely understood, it is tempting to speculate that the numerous and conspicuous mitotic figures that are frequently observed in mod-
erately well-differentiated squamous cell carcinomas may reflect a slow process of mitosis, whereas mitotic divisions are more rapidly completed in the cells of poorly differentiated tumors. Last but not least, given that low-grade cancers have a tendency to differentiate, a substantial number of postmitotic cells will not resume proliferation and become Ki-67 negative, whereas cells of high-grade carcinomas may retain their proliferative potential after completing a division and immediately re-enter the reproductive cycle. This would explain an inverse variation in the global growth fraction as compared with the mitotic index in well-differentiated and poorly differentiated cancers (23).

The observed mean Ki-67 labeling index in the present study was 8.47±1.586 in healthy oral mucosa, 25.5±3.01 in leukoplakia with mild dysplasia, and 29.7±3.04 in well-differentiated squamous cell carcinoma.

A report by Macluskey et al. (1999) found that the correlation with the mean Ki-67 labeling index was stronger for the healthy tissue and dysplasia than for carcinomas, but was significant in all three cases: 3.1±3.1 for healthy oral mucosa, 20.2±11.1 for dysplasia, and 24.8±12.7 for carcinoma. The results suggest that epithelial proliferation may continue to increase during the transition from dysplasia to carcinoma, but this is likely to occur at a slow rate and Ki-67 expression is not a good indicator of neoplastic transformation (24).

One more reason for the increase in proliferating cells with Ki-67 has been explained by Slootweg et al., who found that tumor cells are in some way able to bypass the suppressive action of the p53 gene. It is concluded that the appearance of p53 protein occurs early in carcinogenesis and that cells also may show increased proliferation without involving immunohistochemically detectable alterations in the p53 gene (22).

It was found that there is a decrease in cells undergoing apoptosis in less-differentiated tumors with an increase in the number of tumor cells and of tumor growth. This decrease in apoptosis was correlated with an increase in the proliferative activity by Hindermann et al. (25).

In the present study, proliferative activity was found to be increasing in lesions with associated habits in premalignancy and malignancy but this increase was found to be statistically insignificant. The reason may be the fact that squamous epithelium exposed to tobacco very frequently responds with an increased epithelial thickness corresponding to a higher cellular turnover rate and increased proliferation.

The reason for such finding has been explained by Wederberg et al. (2), who indicate that the p53 gene is involved in the initial events leading to subsequent malignant transformation of oral mucosa exposed to snuff. Furthermore, mutations of the p53 gene have been associated with increased proliferation with greater risk of perpetuation of mutations and malignant transformation. They also found that the mean age of the patients with a snuff habit was 39.5±12.5 years.

Liu et al. (9) showed no significant influence on the results with MIB-1 by age, sex, and smoking habits. However, in the present study, while there were insignificant differences in proliferative activity with respect to age in both premalignancy and malignancy, a statistically significant difference was found with sex in both.

Roland (26) did not find any correlation between the Ki-67 index and the host factors, such as age and sex. Porschke et al. reported that the Ki-67 index was independent of sex and age in patients with colorectal carcinoma (27).

According to Macluskey et al., the proliferative indices of dysplastic and carcinomatous tissues did not show a significant difference when analyzed with the possible association between proliferative index and the use of tobacco as well as age and sex of the patients (24).

Sittel et al. found that the proliferative index was higher for men and people more than 50 years of age (28). Lim et al. reported that there was no correlation between proliferative index and factors like age, sex, and tumor location (29).

Liu et al. showed that age, sex, and smoking habits
did not influence MIB-1 results (9).

Such great variability may be the result of many factors, including selection of cases, size of samples, number of cells counted, evidence of reproducibility, and methods of statistical analysis used. Other possible variables include sample size, anatomical site, circadian fluctuation in proliferative index, fixation, and antigen retrieval technique (30, 31).

The distributional disturbance of proliferating cells and suspected stem cells in oral epithelial dysplasia presented in this study suggest a loss of function of stem cells, because cancer stem cells could contribute to the development of oral epithelial dysplasia and oncogenesis of oral squamous cell carcinoma (13).

The proliferative changes in premalignant lesions in the head and neck region suggests the degree of proliferative dysregulation might be used as a prognostic marker for revealing the highest risk of progression to overt carcinoma.

In conclusion, we can suggest that the novel antibody MIB-1 represents a robust and easily applicable marker of cell proliferation that may be employed in a wide range of archival histological materials and whose expression correlates well with the disease progression from dysplasia to carcinoma. This method should be regarded as a useful adjunct to conventional histological techniques, allowing more objective grading of benign and malignant epithelial lesions.

References