SUPRABASAL EXPRESSION OF Ki-67 ANTIGEN AS A MARKER FOR THE PRESENCE AND SEVERITY OF ORAL EPITHELIAL DYSPLASIA

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Abstract: *Background.* Suprabasal expression of Ki-67 is assessed as a marker for oral dysplasia. The study involved non-neoplastic epithelium adjacent to 74 oral squamous cell carcinomas.

Methods. An immunohistochemical technique was carried out (peroxidase-antiperoxidase) with the monoclonal antibody MIB-1. Epithelial expression of Ki-67 was classified as being absent, basal, and suprabasal. The epithelium was normal in 19 cases, hyperplastic in 38 cases, and dysplastic in 37 cases. The dysplasia was slight in 20 cases, moderate in 12 cases, and severe in 5 cases.

Results. The results of the expression of Ki-67 were in normal epithelium, basal expression 9 cases, absent 10 cases; in hyperplastic epithelium, basal expression 18 cases, absent 20 cases; in dysplastic epithelium, basal and suprabasal expression (always jointly) 27 cases, absent 10 cases; all the severe and moderate dysplasia cases expressed suprabasal Ki-67. A significant association was observed between the presence ($p < .0001$) and severity ($p < .007$) of the dysplasia and the suprabasal expression of Ki-67. © 2000 John Wiley & Sons, Inc. *Head Neck* **22:** 658–661, 2000.

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The development of head and neck cancer (HNC) has been proposed as a multistep process derived by an accumulation of cellular alteration resulting from carcinogen exposure.[1] It has been postulated that the accumulation of molecular and genetic alterations can initiate phenotypic changes in the tissue, which can be recognized histologically and could be associated with a dysregulation of cell proliferation and differentiation.[2] The various steps presumed to play a role in HNC tumorigenesis are epithelial hyperplasia, epithelial dysplasia, and HNC. It is hypothesized that only those cells with high proliferative activity could be associated with premalignant tissue changes during carcinogenesis.[3]

Different methods have been used for studying cell kinetics in proliferative specimens. These include (1) tritiate thymidine incorporation followed by autoradiography; (2) bromodeoxyuridine (BrdU) incorporation followed by antiBrdU immunocytochemistry; (3) flow cytometric analysis; and (4) immunocytochemistry using cell cycle specific monoclonal antibodies.[4] This last method has important advantages such as preservation of tissue architecture, the fact that it does not require

fresh tissue or radioactive substances, and that can be used in paraffin-embedded material.[5] Among the monoclonal antibodies used in the study of cellular kinetics there are those that recognized the proliferating cell nuclear antigen (PCNA) and the Ki-67 antigen. PCNA is not expressed throughout the whole cellular cycle. It is first detected in the G1 phase, reaches its maximum expression in the S phase or in G1/S, it falls in the G2 phase, and becomes impossible to detect by immunohistochemical methods in M phase.[6] Ki-67 antigen is expressed in proliferating cells (G1, S, G2, and M phases) but not in resting cells (G0 phase).[7]

It is well known that the basal layer of the oral epithelium is the location of the normal proliferating cell compartment, whereas suprabasal layers are only spaces of cellular maturation whose cellular alterations show potential signs of dysplasia.[8] Because the diagnosis of epithelial dysplasia and the evaluation of its severity is performed by the subjective interpretation of different histologic facts, some markers of cellular proliferation may help to establish a more objective evaluation of the presence and severity of epithelial dysplasia in the oral mucosa.[9]

The objective of this study was to evaluate the effectiveness of the suprabasal expression of Ki-67 as a marker of the presence and severity of the epithelial dysplasia in the non-neoplastic epithelium adjacent to squamous carcinomas in the oral cavity, where presumably the first step was the appearance of dysplasia and subsequently the tumor developed.[10]

MATERIALS AND METHODS

A study was carried out on a group of 74 patients, 68 men and 6 women aged between 40 and 85 (mean age, 62.4), diagnosed and treated for squamous cell carcinoma at the University Hospital of Granada (Spain). In all cases, an assessment of the clinical history was made. In addition, a section of the paraffin block of the primary tumor was obtained and stained with H & E, which was used for the histopathologic analysis of the non neoplastic epithelium adjacent to the tumor, in which the presence of epithelial dysplasia and its severity were assessed (with the categories of dysplasia classified as mild, moderate, and severe, according to the criteria of the pathologist), the presence of epithelial hyperplasia, and the existence of a normal epithelium.

For immunohistochemical detection of Ki-67, 4-μm sections were cut from formalin-fixed paraffin-embedded mucosa samples. The sections were deparaffinized by xylene and incubated in 96% ethanol. Endogenous peroxidase activity was blocked by incubating the slides in methanol with 1.5% H_2O_2 for 20 min. For antigen retrieval, the sections were boiled in citrate buffer (2.94 g/L sodium citrate, pH 6.0) for 15 min and subsequently cooled to 30°C. After washing with phosphate-buffered saline (PBS), the sections were incubated with 10% nonimmune horse serum for 15 min. Subsequently, they were incubated for 60 min with 1:200 diluted mouse-anti-Ki-67 (MIB-1, Immunotech, S.A., Marseilles, France) followed by washing in PBS and incubation with 1:500 diluted biotinylated horse antimouse antibody (Vector Laboratories Inc., Burlingham, CA) for 30 min. After washing in PBS, the sections were incubated for 30 min with peroxidase-streptavidin conjugase (Immunotech) in a 1:400 dilution. The sections were washed in phosphate-citrate buffer (17.5 g/L $Na_2HPO_4 \cdot 2\ H_2O$ and 10.7 g/L citric acid, pH 5.8) and Ki-67 was visualized for light microscopy with DAB reagent (0.06 % 3,3-diaminobenzidine tetrahydrochloride and 0.03% H_2O_2 in phosphate-citrate buffer).

Sections were counterstained with hematoxylin for 3 min. The expression of Ki-67 antigen was classified as absent, basal, and suprabasal. Both the histopathologicl evaluation of the non-neoplastic epithelia adjacent to the tumor and that for the expression of Ki-67 were carried out by the same pathologist.

To contrast the expression with type and intensity of dysplasia the chi-square test and the Fisher test were used. All data were processed with SPSS Program for Windows, version 6.01 (SPSS Inc, Chicago, IL).

RESULTS

In Table 1, the distribution of the presence and location of the expression of Ki-67 may be observed according to the type of epithelium considered. It may be verified that the basal and suprabasal expression of Ki-67 (always jointly) was significantly more frequent ($p < .001$) in the epithelia with epithelial dysplasia (Fig. 1).

Table 2 shows the distribution for the cases according to the presence and location of the expression of Ki-67 and the intensity of the epithelial dysplasia. The suprabasal expression of Ki-67 was significantly more frequent ($p < .01$) as the intensity of the dysplasia increased.

No significant association was observed between the pattern for the expression of Ki-67 in

Table 1. Relationship between the expression of Ki-67 and the type of epithelium

	Type of epithelium			
	Normal *n* (%)	Hyperplastic *n* (%)	Dysplastic *n* (%)	Total
Expression of Ki-67				
Negative	10 (52.6)	20 (52.6)	10 (27.0)	40
Basal	9 (47.4)	18 (47.4)	0 (0)	27
Basal suprabasal	0 (0)	0 (0)	27 (73)	27
Total	19	38	37	94

χ^2 *(4 gl) = 62.58* p < *.001.*

the non-neoplastic epithelium adjacent to the tumor and the location of the tumor, the age, and the sex of the patient.

DISCUSSION

Certain studies have been carried out that attempt to determine whether the expression of the antigen Ki-67 is in any way related to the presence of epithelial dysplasia. So, Kushner et al[11] carried out a study into epithelial dysplasia in the floor of the mouth in which they observed that the proliferative indices (number of cells that express Ki-67 per millimeter of the membrane basement) were significantly greater as the degree of epithelial dysplasia increased. Along the same line, Zoeller et al,[12] when studying epithelial dysplasia and carcinoma in the oral cavity, observed that the percentage of Ki-67–positive cells increase in accordance with the histopathologic degree of malignancy. Saito et al[13] found that expression of Ki-67 increased according to the cell proliferative activity. In other areas in the upper aerodigestive tract (UADT) the expression of Ki-67 has also been evaluated as a marker of epithelial dysplasia. Hong et al,[7] when studying the proliferative compartment in the Barrett's esophagus, observed that the percentage of Ki-67–positive nuclei correlated to the degree of dysplasia, so the authors concluded that the pattern of expression of the Ki-67 may represent an additional parameter for distinguishing between patients with or without dysplasia in the case of Barrett's esophagus. Although other studies[14] have demonstrated the value of the suprabasal expression of Ki-67 in other locations of the UADT as a marker of epithelial dysplasia, our study is the first to approach how to determine the value of the suprabasal expression of Ki-67 as a marker of the presence and severity of epithelial dysplasia in the oral mucosa.

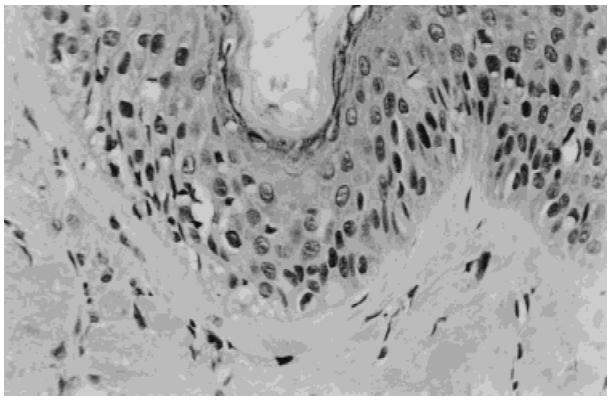

FIGURE 1. Basal and suprabasal expression of Ki-67 in an oral epithelium with epithelial dysplasia (stain, H & E; original magnification ×40).

The basal layer is the only proliferative compartment for normal oral epithelium, whereas in the rest of the epithelial layers, cellular maturation is only produced without any proliferative activity.[8] Hence, any sign of proliferative cellular activity beyond the basal layer should be considered as a warning sign. Epithelial dysplasia is characterized by a number of cell and tissue alterations, visible with a light microscope, that reveal an alteration of the cellular maturation in the epithelium and an increase of the proliferative suprabasal activity (for example, presence of mitosis in the upper half of the epithelium). Nevertheless, these cellular alterations are assessed by pathologists who inevitably use a certain degree of subjectivity when classifying the presence and severity of the epithelial dysplasia. It has often been observed that different pathologists issue different reports about the same sample of dysplastic epithelium.[8]

Because the factor that has the most influence on the potential for malignant transformation of a premalignant lesion is the presence and severity of the epithelial dysplasia,[10] it is therefore essen-

Table 2. Relationship between the expression of Ki-67 and the intensity of the epithelial dysplasia

	Intensity of the dysplasia		
	Mild *n* (%)	Moderate + severe *n* (%)	Total
Expression of Ki-67			
Negative	10 (33.3)	0 (0)	10
Basal suprabasal	20 (66.6)	17 (100)	37
Total	30	17	47

p < *.01.*

tial to find objective markers for the presence and severity of the epithelial dysplasia. Along the same line, we have observed in this study that the expression of Ki-67 had a significant correlation to the presence and severity of the epithelial dysplasia in the nontumoral epithelium adjacent to a squamous carcinoma in the oral cavity.

We have previously reported[15] that the suprabasal expression of PCNA is an objective marker of epithelial dysplasia. Similar results have been found by other authors.[1,3] However, PCNA is only detectable using immunohistochemical methods during the S or G1/S phase of the cell cycle, whereas it is impossible to detect during G2 and M phases.[7] This means that some cells in advanced phases of the cell cycle do not express PCNA and cannot be detected by means of immunohistochemical techniques that demonstrate this antigen from cell proliferation. On the other hand, the Ki-67 antigen is expressed in all the phases of the cellular cycle in proliferative cells, whereas it is not expressed in quiescent cells (G0 phase)[7]. So, probably, the suprabasal expression of Ki-67 could well be a more reliable marker of epithelial dysplasia than the suprabasal expression of PCNA, although this hypothesis will have to be confirmed in future studies.

Van Oijen et al[16] observed an increase in the number of proliferative cells, measured by Ki-67 expression, in the epithelium adjacent to squamous carcinoma of the oral cavity in patients who are smokers, whereas this increase was not seen to the same extent in the nontumoral epithelium adjacent to squamous carcinoma in the oral cavity of patients who are nonsmokers. The authors highlight the fact that smoking leads to an increase in the number of proliferative cells in the UADT. However, they did not evaluate the location of the positive Ki-67 cells in the said epithelia and their relationship to the presence and severity of the epithelial dysplasia, although the increase of proliferative cells observed by these authors is probably carried out also at the expense of cells located in suprabasal layers. Our results are not comparable with those attained by van Oijen et al[16] because all our patients with oral cancer were smokers.

To summarize, the immunohistochemical differences found between dysplastic epithelia and nondysplastic ones adjacent to squamous cell carcinomas in the oral cavity support the usefulness of the suprabasal expression of Ki-67 as an objective marker for the presence of the epithelial dysplasia of the oral mucosa and show a grater frequency of suprabasal expression of Ki-67 as the severity of the dysplasia increases.

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