Expression of P16, P53 and Ki-67 Proteins in the Progression of Epithelial Dysplasia of the Oral Cavity

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Abstract. *Background: The overexpression of the protein products of genes associated with the cell cycle tumour protein53 (p53), cyclin-dependent kinase inhibitor 2A (p16) and antigen identified by monoclonal antibody Ki-67 (Ki-67) is apparently of great significance. This study evaluated the immunohistochemical expression of these proteins in precancerous lesions and in carcinoma of the oral cavity. Materials and Methods: The nuclear expression of p53 and Ki-67 and nuclear and/or cytoplasmic expression of p16 protein was examined in 54 biopsy specimens from the oral cavity obtained over a period of 3 years. The samples included 18 cases of normal/hyperplastic mucosa, 25 cases of dysplasia and 11 cases of invasive squamous cell carcinoma. The specimens were grouped into three categories: 1 = no or mild dysplasia, 2 = moderate or severe dysplasia, and 3 = invasive carcinoma. Results: p16 was negative in all the group 1 specimens, while both p53 and Ki-67, when present, were limited to the cells of the basal layer. In the group 2 specimens, the number of p16-, p53-, and Ki-67-positive cells increased as the grade of dysplasia progressed. In group 3 (invasive carcinomas), p53 and p16 expression occurred respectively in 81.8% and 54.5% of cases, while Ki-67 was elevated in all the cases. Conclusion: The expression of the cell-cycle proteins p16 and p53 in the dysplastic epithelium, in association with Ki-67, may represent significant markers to recognize evolution of precancerous disease in the oral cavity and to improve identification of the degree of dysplasia.*

Carcinoma of the oral mucosa is the end-result of a multistep process involving several genetic modifications, which in most cases occur early, often preceding morphological alteration of the epithelium. Morphological evaluation of epithelial dysplasia of the oral cavity is subject to wide inter-observer variability in grading due to several factors, of which the presence of inflammation, lesion site and biopsy technique predominate, since they can alter the reliability of histological diagnoses (1). Thus diagnosis is often subjective, and does not respond to strict criteria, as it does, for example, in the uterine cervix. Hence grading is not always predictive of a possible evolution of the disease (2, 3). In the oral mucosa, the different degrees of dysplasia represent the morphological steps of epithelial transformation, but in only a few cases does dysplasia evolve into carcinoma, and no specific markers exist that can identify the probability of progression (4). Recent studies have hypothesized that inactivation of some tumour suppressor genes, namely TP53 (tumour protein 53, p53) and CDKN2A (cyclin-dependent kinase inhibitor 2A), may play a significant role in oral carcinogenesis (3, 5).

TP53, a tumour-suppressor gene located on the short arm of chromosome 17, encodes a protein involved in preventing accumulation of damaged DNA, consequently triggering cell apoptosis (6-8). Due to this characteristic, it has been called the "guardian of the genome" (9). Alterations affecting the *TP53* gene produce accumulation of an anomalous protein in the nucleus, which can be highlighted by immunohistochemistry. Some studies have indicated that, in the oral cavity, the presence of p53-positive cells in the upper layers of the dysplastic epithelium may imply a risk of progression to carcinoma (3). The combined study of the cell proliferating index Ki-67 and p53 has been widely exploited in defining dysplasia in various types of epithelium (*e.g.* larynx, urothelium, *etc.*) and is thus both a valid diagnostic tool, compared to haematoxylin-eosin (H-E) alone and a help in understanding the pathogenesis of the dysplasia-carcinoma sequence.

CDKN2A, a tumour suppressor gene located on chromosome 9p21, encodes a cell-cycle protein (p16) which is a strong and specific inhibitor of cyclin-dependent kinases (CDK) 4 and 6 and down-regulates cyclin D-dependent

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Table I. *Location and diagnosis of oral biopsies.*

Site of biopsy	Group 1 No/Mild D (%)	Group 2 Mod D/Sev D (%)	Group 3 SqCC (%)
Buccal mucosa	6 (11.1)	6 (11.1)	3 (5.5)
Mouth floor	2 (3.7)	2 (3.7)	3 (5.5)
Lip	1 (1.9)	1 (1.9)	1 (1.9)
Gingiva	12 (22.2)	0 (0)	0 (0)
Tongue	8 (14.8)	5 (9.2)	4 (7.4)
Total	29 (53.7)	14 (25.9)	11 (20.4)

No=Normal mucosa; SqCC=invasive squamous cell carcinoma; mild D=mild dysplasia; Mod D=moderate dysplasia; Sev D=severe dysplasia.

Table II. *Summary of immunoexpression of Ki-67, p53 and p16 in precancerous disease and in invasive squamous cell carcinoma of the oral cavity.*

Group	Ki-67	p53	p16
1 (n=29): No or mild dysplasia	0 (0%)	2 (6.9%)	0 (0%)
2 (n=14): Moderate or severe dysplasia	14 (100%)	9 (64.3%)	12 (85.7%)
3 (n=11): Invasive carcinoma	11 (100%)	9 (81.8%)	6 (54.5%)

Group 1 *vs*. Group 2: $p \leq 0.001$; Group 1 *vs*. Group 3: $p \leq 0.001$; Group 2 *vs*. Group 3: *p*=n.s.

phosphorylation of the retinoblastoma (Rb) protein, thus blocking cell-cycle progression from the G1- to the S-phase (10, 11). This may lead to a disappearance of the protein in neoplastic cells (12). The study of p16 has to date been almost entirely limited to pre-cancerous lesions of the uterine cervix, as a "surrogate" to identify cells infected by the high risk human papilloma virus (HR-HPV) carried by the protein E7, which inhibits the anti-oncogene Rb, in its turn linked to p16 through a negative feedback. However, the significance of accumulation of p16 protein in the dysplastic lesions of the upper aerodigestive tract is controversial and poorly understood (13, 14).

This study investigated the immunohistochemical expression of p53, p16 and Ki-67 proteins in dysplastic lesions and in invasive squamous cell carcinoma of the oral cavity, as a potential biological marker for grading dysplasia.

Materials and Methods

Cases. Fifty-four formalin–fixed, paraffin-embedded biopsy specimens of the oral cavity obtained by the Milan University Pathological Anatomy Institute from April 2005 through March 2008 were evaluated. A representative block for each case was selected and multiple 5 μm sections were cut. The first and the last sections, stained with H-E were evaluated by two investigators (FA, ED). If there was no agreement, which occurred in four cases of dysplasia (16%), the H-E slides were re-evaluated using a multiheaded microscope until consensus was achieved. The biopsies included 25 dysplasias (14 men, 11 women, ranging in age from 44 to 84 years) and 11 invasive squamous cell carcinoma specimens (8 men, 3 women, ranging in age from 50 to 80 years). Dysplasia was classified as mild, moderate or severe following the WHO tumour classification (15). The remaining 18 specimens included cases with normal or hyperplastic mucosa (10 men and 8 women, ranging in age from 8 to 73 years). The specimens, as suggested by others (3), were divided into three categories: group 1 consisted of 29 patients with normal/hyperplastic mucosa or mild dysplasia; group 2, consisted of 14 specimens with moderate or severe dysplasias and group 3 included 11 specimens with invasive carcinomas (Table I).

Immunohistochemistry. Immunohistochemical analysis was performed on paraffin sections within 4-6 weeks of sectioning in order to maintain their antigenicity. The sections were incubated using the avidin-biotin-peroxidase complex with the following antibodies: p53 (clone DO7, dilution 1:150; Neomarkers, Union City, CA, USA), Ki-67 (clone MIB-1, dilution 1:50; Dako, Copenhagen, Denmark), p16 (clone E6H12, dilution 1:40; Novocastra, Newcastle, UK). For each antibody, positive and negative controls were used. Two independent observers (ED, FA) reviewed the immunohistochemically-stained sections. In groups 1 and 2, Ki-67 and p53, according to Cruz *et al*. (3) and to Pirog *et al*. (16) were classified as positive or negative, attributing a score taking into account both the relative number of stained nuclei and their localisation in the upper 2/3 of the epithelium. In the invasive carcinomas (group 3), evaluation was made in the areas of infiltration, where cell proliferation was more active. With regard to expression of the protein p16, immunostaining was considered positive when at least 10% of nuclei were stained (17).

Statistical analysis. Statistical analysis was performed using the Chi-square test. The difference in values was considered significant when $p<0.05$.

Results

The sources of the 54 oral biopsies are shown in Table I. Consensus histological diagnosis was reached in all cases. Classification of the oral biopsies was as follows: 25 cases of dysplasia (11 mild, 10 moderate and 4 severe dysplasia), 11 cases of invasive squamous cell carcinomas. The 18 cases of non-dysplastic mucosa consisted of normal mucosa and epithelial hyperplasia (13 and 5 cases, respectively).

In all the cases of mild dysplasia and in the non-dysplastic cases (group 1), immunostaining for Ki-67 was limited exclusively to the lower third of the epithelium (Figure 1A) and was thus considered negative. Clusters of immunoreactive nuclei in the upper 2/3 of the epithelium (positive cases) were significantly correlated ($p<0.001$) with the group 2 cases (moderate and severe dysplasia) (Figure 1B), and in the areas of infiltration of all the carcinomas (Figure 1C) (Table II).

The immunohistochemical p53 expression in the nuclei of the lower layers was considered normal (Figure 2A), while expression in the upper layers was detected in 2 cases (6.9%) of group 1, in 9 cases (64.3%) out of group 2 (Figure 2B) and in 9 cases (81.8%) out of invasive carcinoma (group 3) (Figure 2C). Immunohistochemical expression of p53 increased as the severity of the lesion increased and was significantly associated with group 2 and group 3 cases ($p<0.001$) (Table II).

The immunohistochemical expression of p16 increased with the degree of dysplasia. Absent from the group 1 cases (Figure 3A), it was closely associated with group 2 (moderate/severe dysplasia) in 85.7% of cases ($p<0.001$) (Table II). The clear-cut demarcation of the stained cells (corresponding to areas of dysplasia) *versus* the contiguous unstained normal epithelium was evident (Figure 3B). In group 3, expression of p16 was present in 54.5% of the areas of carcinoma infiltration ($p<0.01$) (Figure 3C) (Table II).

Discussion

The immunohistochemical expression of Ki-67 and p53 showed similar trends, and increased with the degree of dysplasia. The expression of Ki-67 was significant in differentiating normal mucosa, benign diseases and mild dysplasia (group 1), on one hand, from moderate and severe dysplasia (group 2) on the other, but it appeared to be less useful in differentiating these conditions from invasive carcinoma (group 3). With regard to the expression of p53, a progressive and significant increase was observed from mild dysplasia to infiltrating carcinoma, in agreement with other studies (18, 19).

p16 Protein, widely studied in precancerous lesions and in carcinoma of the uterine cervix, has, in the past, often been correlated with progression of dysplasia in HR-HPV infection and is now considered an important marker of transformation in both histological sections and cytological specimens (20). With regard to the oral cavity, little is certain either of the significance of p16 expression in pre-malignant squamous epithelium (10, 15), or of the step at which it becomes expressed (21). Opinions are far from being unanimous; some studies have shown an increased expression of p16 as the degree of dysplasia progresses (22), while others have found an inverse correlation (23, 24). Some authors have suggested that p16 immunohistochemistry was not helpful in differentiating dysplastic from non-dysplastic oral mucosa, so was not a reliable marker in routine clinical practice (25). Furthermore, it has been found that the expression of p16 in oral lesions was independent of the presence of HR-HPV (13, 21), but the opposite has also been suggested (23, 24).

In the present study, p16, which was absent from the normal and mild dysplasia specimens (group 1), became progressively more expressed in moderate/severe dysplasia

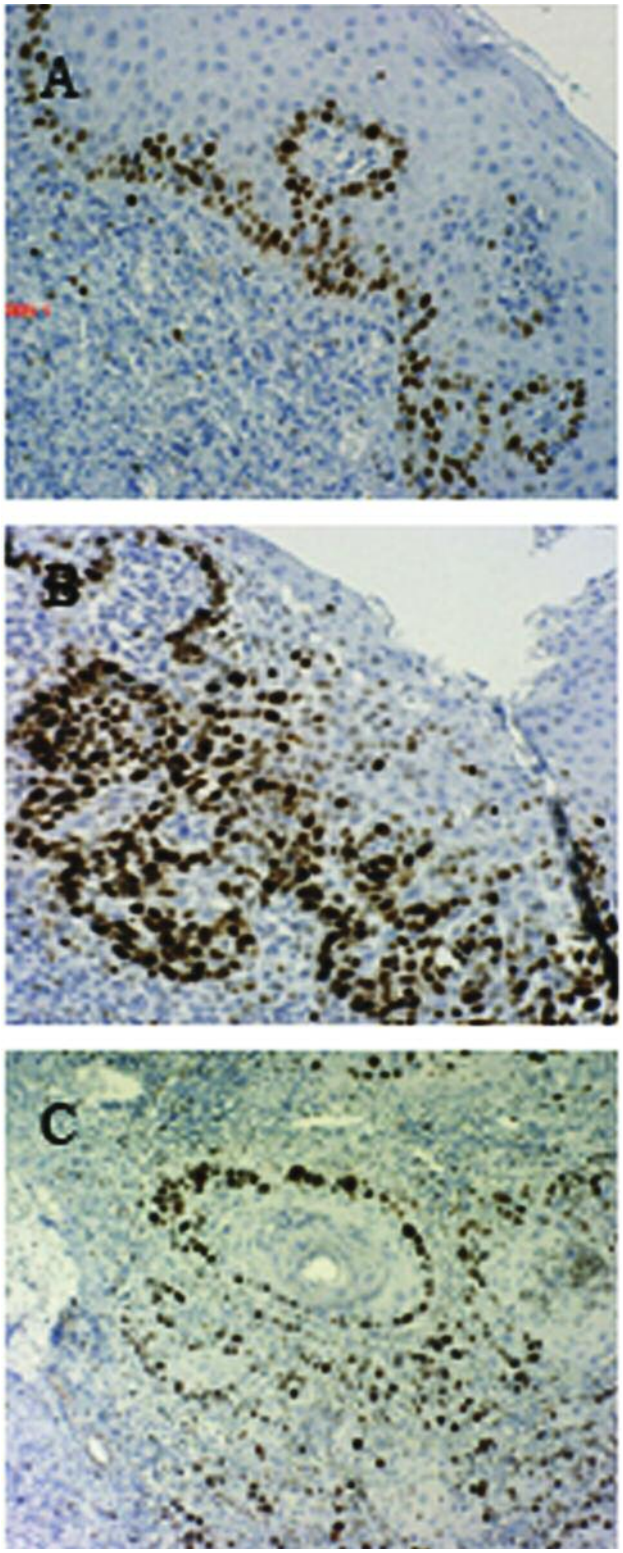

Figure 1. *Immunohistochemical findings of Ki-67 expression. A, Immunostaining limited exclusively to the lower third of the epithelium. B, Clusters of immunoreactive nuclei in the upper 2/3 of the epithelium with moderate and severe dysplasia. C, Immunostaining strongly positive in the areas of carcinoma infiltration.*

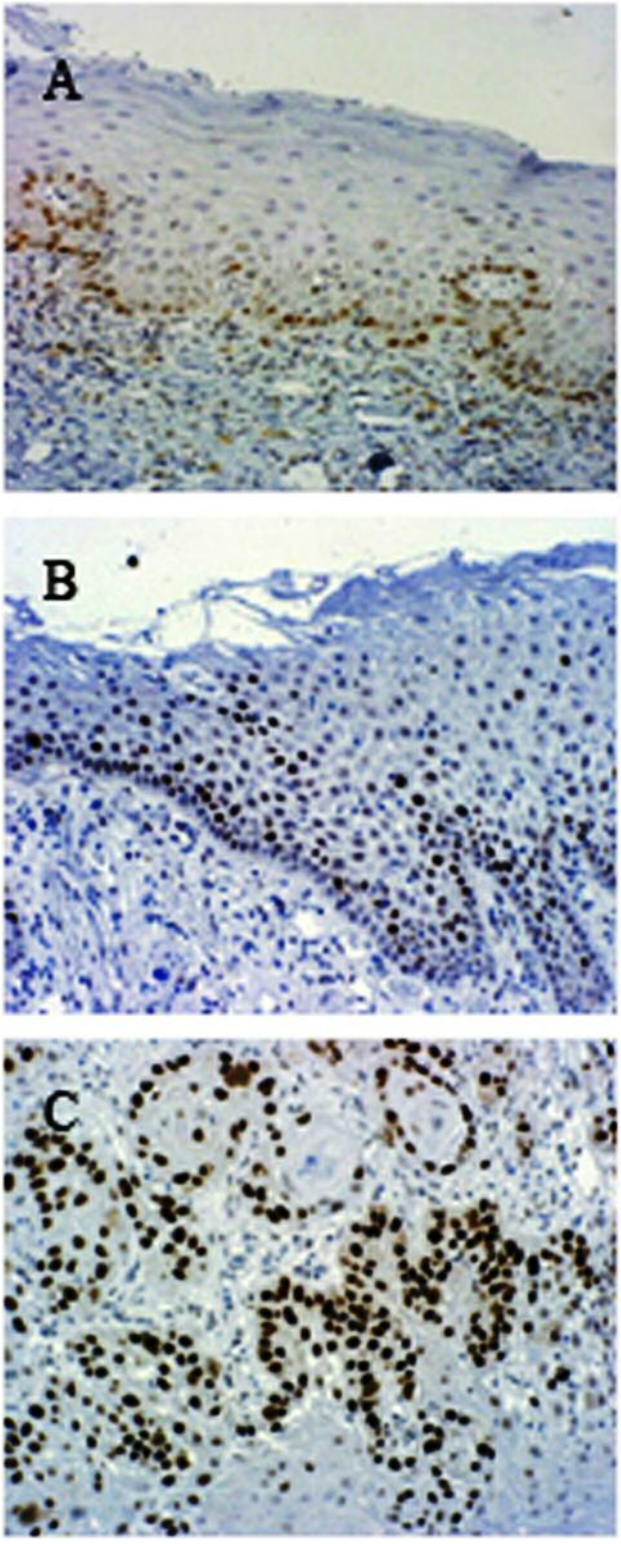

Figure 2. *Representative immunohistochemical patterns of p53. A, Positive expression of p53 protein only in the lower layers of the epithelial cells. B, Positive immunoreactivity in areas of moderate to severe dysplasia. C, Immunostaining strongly positive in areas of carcinoma infiltration.*

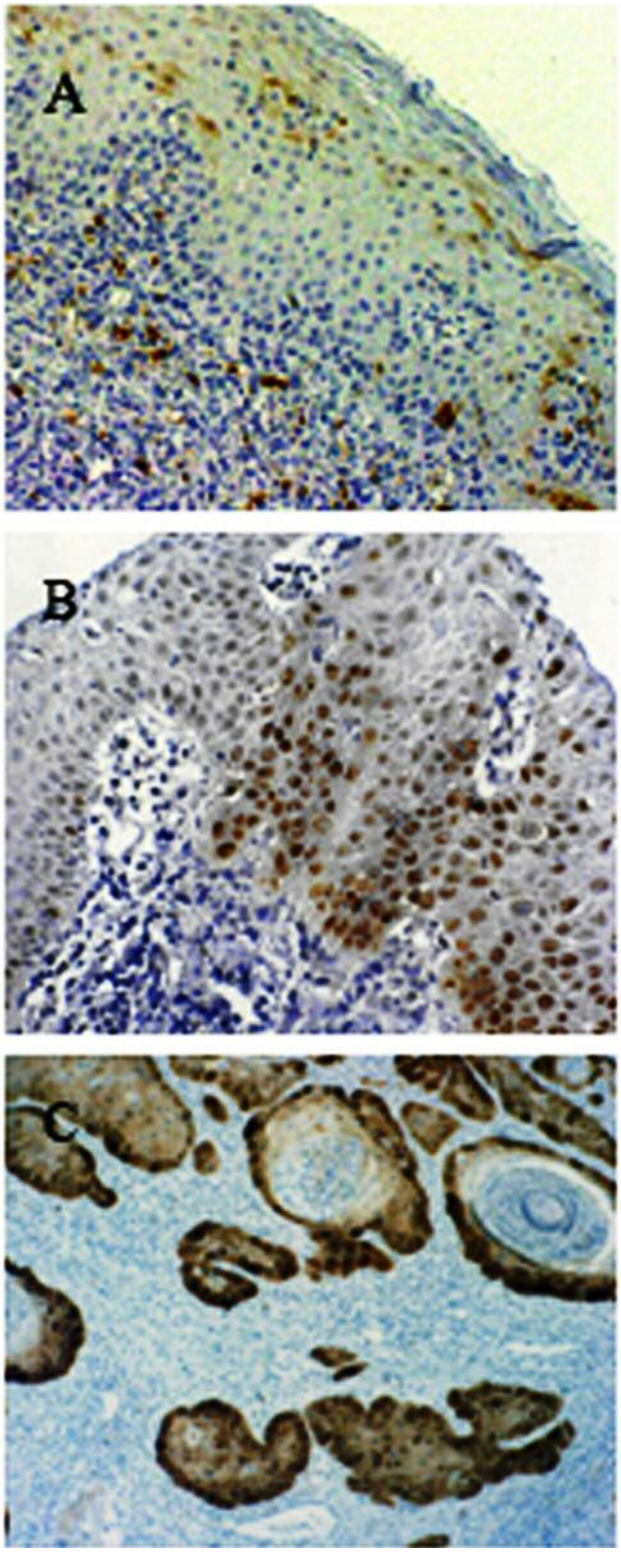

Figure 3. *Immunohistochemical expression of p16 protein. A, Expression absent in a group 1 case. B, Clear-cut demarcation of the stained cells (corresponding to areas of dysplasia) versus the contiguous unstained normal epithelium. C, Expression of protein p16 present in the areas of carcinoma infiltration.*

(group 2) (85.7%). In invasive carcinoma (group 3), however, it was present in only 6 out of 11 cases (54.5%). Our findings were similar to those observed in the uterine cervix, where the overexpression of p16 has been demonstrated as the degree of dysplasia increases and resulted from high-risk HPV infection. The present results, in agreement with some authors (13, 21), greatly differed from others (2, 25). This was probably due to the different methodology, antibodies utilized and criteria adopted.

However, we find of significance that the trend of expression of p16 may reflect the biomolecular pathway to which it is linked (CDK/Rb), in itself not linked by the usual immunophenotypical and morphological profile of the progression of dysplasia.

In conclusion, an increased expression of the cell-cycle proteins p16 and p53, together with the proliferation index marker Ki-67, provides significant help in grading oral cavity dysplasia. However, further studies investigating HR-HPV status and *CDKN2A* and *TP53* gene inactivation will be needed to confirm the importance of overexpression of the p16 and p53 proteins in predicting the evolution of dysplasia into invasive carcinomas of the oral cavity.

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