Immunohistochemical expression of p16\(^{INK4A}\) protein in oral lichen planus

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The expression of p16\(^{INK4A}\) has been investigated in oral leukoplakias (OLK), but no data are available about oral lichen planus (OLP). In this study, p16\(^{INK4A}\) immunohistochemical expression was evaluated in 56 OLP and 36 OLK (12 without inflammation [NI-OLK] and 24 with chronic inflammation [I-OLK]) and compared with 23 reactive nonspecific inflammations (INF) and 14 normal control samples. The p16\(^{INK4A}\) immunostaining was considered to be positive when >5% of keratinocytes were stained. All normal control samples were negative. Positive p16\(^{INK4A}\) was detected in OLP, IOLK, and INF. Significant differences in p16\(^{INK4A}\) positivity were found between OLP (64%) and OLK (28%) (\(\chi^2 = 17.7; P < .01\)), and between I-OLK and NI-OLK (\(\chi^2 = 4.5; P < .05\)). No significant difference was found between OLP and INF (43%). In conclusion, positive p16\(^{INK4A}\) in OLP patients seems to be related to reactive inflammatory processes rather than to a risk of progression to oral squamous cell carcinoma. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:222-227)

Oral squamous cell carcinoma (OSCC) arises through a multistep process of cumulative genetic alterations leading to a loss of cell cycle control.\(^1\) Loss of short arm of chromosome 9 is one of the more common and earlier genetic abnormality in malignant development.\(^2-4\)

The p16\(^{INK4A}\) protein, which gene maps on 9p21, functions as negative regulator of the cell cycle progression through its inhibition of CDK4 and CDK6 kinases and subsequent blockage of retinoblastoma (Rb) cyclin-dependent phosphorylation.\(^6\) Genetic alterations of p16\(^{INK4A}\) lead to a deregulation of the restriction point in the G1 phase of the cell cycle and promote cellular transformation.\(^7-9\)

Data regarding the expression of p16\(^{INK4A}\) in OSCC are controversial: reduction of p16 \(^{INK4A}\) expression with consequent loss of function is frequently observed,\(^9-11\) but paradoxically, several authors reported p16\(^{INK4A}\) overexpression in 13%-50% of OSCC.\(^6,10,12-14\)

Conflicting results are also observed regarding the expression of p16\(^{INK4A}\) in oral preneoplastic lesions. Golongan et al.\(^15\) and Cunningham et al.\(^16\) demonstrated a positive correlation between oral dysplasia and p16\(^{INK4A}\) expression, whereas other studies showed a reduction of p16\(^{INK4A}\) proportionally to the increasing grade of dysplasia\(^17,18\) or stated that p16\(^{INK4A}\) is not helpful in differentiating dysplastic from nondysplastic mucosa.\(^19,20\)

In a previous study, we investigated a series of oral preneoplastic lesions that had been followed by invasive OSCC at the same site after a variable time period. The results showed that the majority of OSCCs expressing p16\(^{INK4A}\) protein arose after oral lesions where a positive staining for p16\(^{INK4A}\) protein had been present, and conversely, most p16\(^{INK4A}\) negative OSCCs were preceded by oral lesions where p16\(^{INK4A}\) protein was not detectable. Afterward, we suggested that a strong immunohistochemical p16\(^{INK4A}\) positivity could be a useful parameter to identify oral lesions with high risk of transformation into p16\(^{INK4A}\) positive OSCC.\(^21\)

The abundance of data on oral preneoplastic lesions was in contrast to the lack of data on other potentially malignant oral lesions, such as oral lichen planus (OLP). OLP is a chronic inflammatory autoimmune disease involving cytotoxic T lymphocytes activity against the epithelial cells. On the basis of several clinical investigations showing that oral cancer can develop on lesions previously diagnosed as OLP,\(^22,23\) the World Health Organization had included OLP among the risk conditions for malignant transforma-
tion, even if it is difficult to evaluate the real risk of malignant transformation in OLP patients. So far, there are no studies in the literature investigating the role of p16INK4A pattern in OLP and testing whether it can be a useful parameter for early detection of lesions at high risk of malignant transformation to OSCC. The aim of the present study was to evaluate the expression of p16INK4A protein in OLP and to compare the results with those found in other preneoplastic and inflammatory lesions.

MATERIALS AND METHODS

One hundred twenty-nine consecutive biopsies from patients who presented to the Department of Oral Sciences of the University of Bologna in the past 2 years constituted the basis of the present study. Histologic diagnoses and immunohistochemistry were performed at the section of Anatomic Pathology of the Department of Hematology and Oncology of the University of Bologna at Bellaria Hospital.

Cases were subdivided as follows: 56 cases of OLP, 37 with reticular form and 19 with atrophic/erosive form; 36 cases presenting as oral leukoplakias, 12 cases without any sign or with minimal signs of nonspecific inflammation (inflammatory cells were focally localized through the chorion and submucosa and infiltrated the epithelium not beyond the first third) and 24 with evident signs of inflammation (numerous inflammatory cells were localized through the chorion and submucosa and infiltrated the epithelium beyond the first third); and 23 cases considered to be nonspecific inflammations, consisting of samples from chronic ulcerative lesions or gingival epulis associated with high degrees of reactive tissue inflammation. Nonspecific inflammation was composed of prominent inflammatory infiltration, comprising mature lymphocytes, plasma cells, and macrophages that deeply infiltrated the epithelium. No features of basal cell damage, such as hydropic degeneration or diskertotic basal keratinocytes (Civatte bodies), were seen. Fourteen samples of normal mucosal epithelium obtained from third molar removal or excisions of benign lesions were used as control samples; no features of mucosal inflammation were observed in any control sample. Table I shows the clinical features of the study population.

All tissues were fixed in 4% formalin and paraffin embedded according to routine protocol. Serial 2-µm sections were obtained from each block and stained with hematoxylin and eosin for histologic evaluation. Histologic diagnoses of preneoplastic oral lesions were performed following the criteria proposed in the World Health Organization blue book.

Leukoplakia was clinically defined as a white patch or plaque that will not rub off and that cannot be characterized as any specific disease. Twelve cases classified as leukoplakias did not show any feature of inflammation, whereas 24 cases showed minimal features of chronic nonspecific inflammation.

The diagnosis of OLP was based on clinical manifestations and histologic features. At the clinical level, OLPs were divided into 2 subtypes: 37 patients characterized by plaque and/or reticular lesions alone, and 19 by atrophic/erosive lesions associated or not with plaque and/or reticular. Histologic features included irregular acanthosis, degeneration of the basal layer of the epithelium, and the presence of a band of lymphohistiocytic infiltrate in the upper chorion composed almost exclusively of mature lymphocytes. The morphologic observation of fungal hyphae and spores attributable to Candida was an excluding criterion for the recruitment of OLP for this study.

Immunohistochemical staining was performed by a Ventana Automatic Stainer (Ventana Medical Sys-

| Table I. Clinical features of the study population regarding p16INK4A positivity |
|---------------------------------|---------------|----------------|-----------------|---------------|
|                                 | Leukoplakia (n = 36) | Oral lichen planus (n = 56) | Samples with nonspecific inflammations (n = 23) |
|                                 | Without or with minimal signs of inflammation (n = 36) | With evident signs of inflammation (n = 24) | Reticular (n = 37) | Atrophic (n = 19) | n = 23 |
| Age, y                          | 44 ± 16 | 64 ± 9 | 59 ± 10 | 54 ± 11 | 60 ± 12 | 62 ± 15 |
| Male/female                     | 7/7 | 3/9 | 11/13 | 11/26 | 3/16 | 10/13 |
| Smoker                          | 3 | 3 | 9 | 8 | 3 | 3 |
| Alcohol consumer                | 1 | 1 | 3 | 4 | 1 | 2 |
| Biopsy site                     | 7 cheek, 3 gingiva, 1 palate, 1 tongue | 12 cheek, 7 gingiva, 3 palate, 2 tongue | 37 cheek | 16 cheek, 3 palate | 10 cheek, 5 gingiva, 2 palate, 6 tongue |
| p16INK4A positivity             | 0a | 0a | 10 (42%)b | 21 (57%)b | 15 (79%)b | 10 (43%)b |

a,b Different letters indicate statistically significant differences between groups.
tems). The monoclonal anti-p16\textsuperscript{INK4A} antibody Clone E6H4, prediluted applied was purchased from MTM Laboratories (Heidelberg, Germany). A positive control, consisting of sections from uterine cervical dysplasia, was added to all batches of slides. Cells were considered to be positive when both cytoplasmic and nuclear staining were evident. Positivity was evaluated as previously described.\textsuperscript{26} Briefly, semiquantitative evaluation was performed by counting the percentage of positive cells in 5 different fields (0.12 mm\textsuperscript{2}) at 40× magnification. Cases showing >5% of positive cells were defined as “positive” in accordance with other studies\textsuperscript{19-21}; all control cases showed values <5%.

**Statistical analyses**

Multiple logistic regression analyses were performed to evaluate any significant relationship between p16\textsuperscript{INK4A} expression in OLP versus samples with nonspecific inflammation, OLP versus leukoplakias, reticular OLP subtype versus atrophic/eroded OLP, leukoplakias with evident features of inflammation versus leukoplakias without or minimal features of inflammation, and OLP versus leukoplakias with minimal features of inflammation; smoking habits and alcohol consumption were forced into the models as confounding factors.

**RESULTS**

Control cases included 7 men and 7 women ranging in age from 17 to 72 years (mean 44 ± 16 years). None of them developed neoplastic lesions during a follow-up period ranging from 10 to 24 months (mean 15 ± 6 months). p16\textsuperscript{INK4A} expression was absent or <5%, and, when present, positivity was limited to the basal layer of the squamous oral epithelium.

OLP cases included 14 men and 42 women ranging in age from 28 to 78 years (mean 56 ± 12 years). On histology, 6 cases presented parakeratosis and 1 orthokeratosis. None of them developed neoplastic lesions during a follow-up period ranging from 12 to 24 months (mean 16.8 ± .6 months). Positive p16\textsuperscript{INK4A} expression (>5%) was detected in 36/56 patients (64%). Twenty-one cases of positive p16\textsuperscript{INK4A} expression (57%) were found among the 37 patients with reticular OLP, and 15 cases of positive p16\textsuperscript{INK4A} expression (79%) were found among the 19 patients with atrophic/eroded OLP. Figure 1 shows p16\textsuperscript{INK4A} low expression in a case of reticular OLP (A) and high in a case of atrophic OLP (B).

Cases with leukoplakia included 14 men and 22 women ranging in age from 44 to 81 years (mean 61 ± 10 years). On histology, all of the cases presented marked hyperkeratosis and hypergranulosis. No dysplasia was seen. None of them developed neoplastic lesions during a follow-up period ranging from 12 to 60 months (mean 26.4 ± 19.2 months). Positive p16\textsuperscript{INK4A} expression (>5%) was detected in 10/36 patients (28%). Ten cases of positive p16\textsuperscript{INK4A} expression (42%) were found among the 24 leukoplakias with evident signs of inflammation, and no case of positive p16\textsuperscript{INK4A} expression was found among the 12 leukoplakias without any sign or with minimal signs of inflammation (Fig. 2).

Samples from tissues with nonspecific inflammation included 10 men and 13 women ranging in age from 33 to 83 years (mean 62 ± 15 years). On histology, 13 cases presented marked hyperkeratosis, 6 parakeratosis, and 4 orthokeratosis. None of them developed neoplastic lesions during a follow-up period ranging from 12 to 72 months (mean 25.2 ± 19.2 months). Positive p16\textsuperscript{INK4A} expression (>5%) was detected in 10/23 cases (43%).

![Fig. 1. p16\textsuperscript{INK4A} immunostaining is low in a case of reticular OLP (A) and high in a case of atrophic OLP (B).](image-url)
A statistically significant difference in the p16\textsuperscript{INK4A} expression was found between OLP and leukoplakias ($\chi^2 = 17.7; P < .01$), although the difference between reticular OLP and atrophic/erosive OLP did not reach significance. A statistically significant difference was also found between leukoplakias with evident signs of inflammation and leukoplakias without or with minimal signs of inflammation ($\chi^2 = 4.5; P < .05$). The inclusion into the regression models of smoking habits and alcohol consumption as confounding factors did not affect statistical significances.

No statistically significant differences were found between OLP cases with either atrophic or reticular lichen and cases with nonspecific inflammation and leukoplakias with significant features of chronic inflammation. Furthermore, statistical analyses did not find any significant difference in the p16 expression between hyperkeratosis and orthokeratinized and parakeratinized mucosa.

**DISCUSSION**

The retinoblastoma pathway is one of the major tumor suppressor mechanisms controlling cell proliferation and senescence.\textsuperscript{5} The protein p16\textsuperscript{INK4A} normally binds to cyclin-dependent kinases 4 and 6, preventing their association with cyclin D1. The inhibition of the CDK 4/6–cyclin D1 complex prevents phosphorylation of the retinoblastoma protein (pRb), which holds it bound to the E2F transcriptional factor, canceling its ability to activate genes required to start DNA replication and help cell cycle progression. An inverse relationship was seen between expression of pRb and p16\textsuperscript{INK4A} in many tumors, including OSCC, suggesting a negative feedback intercontrol.\textsuperscript{6,26}

P16\textsuperscript{INK4A} overexpression has been found in 13%-50% of OSCC,\textsuperscript{6,10,12-14} and has been attributed to human papillomavirus (HPV)–induced OSCC by analogy with cervical squamous carcinoma, where the functional inactivation of pRb by the HPV oncoprotein E7 has been demonstrated to lead to the overexpression of p16\textsuperscript{INK4A} protein.\textsuperscript{27-30}

In a previous study, we demonstrated that the majority of OSCCs overexpressing p16\textsuperscript{INK4A} protein were preceded by oral lesions where a positive staining for p16\textsuperscript{INK4A} protein was present many years before, and conversely that most p16\textsuperscript{INK4A}-negative OSCCs were preceded by oral lesions where p16\textsuperscript{INK4A} protein was not detectable. We postulated that although a negative immunostaining is not informative regarding the risk of developing OSCC, because it could also characterize a normal oral mucosa, a strong p16\textsuperscript{INK4A} positivity can be a useful piece of data to differentiate leukoplakias according to their risk to transform into p16\textsuperscript{INK4A}-positive OSCCs.\textsuperscript{21}

Those data agreed with those from Gologan et al.\textsuperscript{15} and Cunningham et al.,\textsuperscript{16} who found in normal epithelium and in nondysplastic keratotic lesions negative or minimal basal p16\textsuperscript{INK4A} immunostaining, whereas a strong immunoreactivity for the p16\textsuperscript{INK4A} antibody was shown from dysplastic epithelium samples.

The results of the present cross-sectional study showed that in a series of consecutive leukoplakias, \~28% displayed high p16\textsuperscript{INK4A} expression, and this percentage may agree with the value range reported in the literature regarding the probability of leukoplakia progression to OSCC.
On the other hand, the results obtained in OLP patients are quite surprising and cannot reflect the progression risk to OSCC. The rate of malignant transformation in OLP ranges from 0% to 4%, whereas >60% of our consecutive OLP patients showed high values of p16INK4A.

Considering that high p16INK4A values were observed with similar ratios in both nonspecific inflammation of oral mucosa and in OLP, our data seem to be consistent with the hypothesis that p16INK4A expression in OLP could be influenced by the presence of tissue inflammation.

The role of inflammation in the p16INK4A pathway could also be speculated by the high p16INK4A expression detected in leukoplakias when evident signs of inflammation were present, whereas p16INK4A normal expression was always found in the remaining leukoplakias devoid of inflammation.

Further, atrophic/erosive OLP forms, where the degree of inflammation and concentrations of tumor necrosis factor (TNF)α are higher, showed higher percentage of p16INK4A positivity compared with reticular OLP form, although the difference did not reach the statistical significance.

Actually, no data are present in the literature regarding the relationship between tissue inflammation and p16INK4A pattern, and, to the best of our knowledge, this is the first study that has described p16INK4A pattern in OLP.

The possibility that high p16INK4A expression in OLP is related to tissue inflammation can be explained by recent data that have shown the role of some cytokines involved in the initiation and maintenance of inflammatory processes and interfering with p16INK4A pathway. Among these, TNF-α is a proinflammatory cytokine that plays an important role in immune and host defense responses to various immunologic disorders, including autoimmune diseases. Recent data have pointed to TNF-α as a key cytokine in the initiation and progression of OLP, underlying the great importance of the cytokines in cell-mediated cytotoxicity against epithelial cells.

The link between TNF-α and p16INK4A has been recently pointed out by studies that have shown that prolonged inflammatory stimulation with TNF-α is able to markedly increase the expression of p16INK4A via the p38 mitogen-activated protein kinase pathway. The process has been interpreted as an attempt to limit the proliferation of epithelial cells during an inflammatory process to avoid uncontrolled growth of “malignant-like” epithelial cells which are resistant to senescence. The p16INK4A-related growth arrest mechanism also has been advocated to explain the block of the proliferation of cells that are exposed to an abnormal epithelial-stromal interface after the basal membrane disruption mediated by matrix metalloproteinases during an inflammatory process.

In addition, interferon-γ is another cytokine highly represented in OLP that plays several roles in the initiation and maintenance of the inflammatory process. It has been reported to induce growth arrest and expression in normal human epithelial keratinocytes, and these events have been associated with an up-regulation of the p16INK4A pathway.

**CONCLUSIONS**

The data obtained here suggest that positive p16INK4A in OLP patients may be uninformative about the risk of progression to OSCC, because it might be the consequence of tissue inflammation. Therefore, even if high p16INK4A positivity can indicate a risk of malignant transformation of the oral mucosa, caution should be used when inflammation is present.

**REFERENCES**

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