Expression of the cell cycle regulation proteins p53 and p21^{WAF1} in different types of non-dysplastic leukoplakias

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Received: October 21, 2010 - Modification: August 7, 2011 - Accepted: August 11, 2011

ABSTRACT

Objectives: The aim of this study was to analyze the immunolabeling of two cell cycle protein regulators, p53 and p21^{WAF1}, in non-dysplastic leukoplakias with different epithelial alterations: acanthosis, hyperkeratosis and acanthosis combined with hyperkeratosis, and compare them with dysplastic leukoplakias. Material and Methods: This was a prospective cohort study involving 36 patients with oral homogeneous leukoplakias. Excisional biopsies were performed and the patients remain under clinical follow-up. The leukoplakias were divided into four groups: 6 acanthosis, 9 hyperkeratosis, 10 acanthosis combined with hyperkeratosis, and 11 epithelial dysplasias. Paraffin-embebeded sections were immunostained for p53 and p21^{WAF1}. Five hundred cells from the basal layer and 500 from the parabasal layer were counted to determine the percentage of positive cells. A qualitative analysis was also carried out to determine the presence or absence of immunohistochemical staining in the intermediate and superficial layers. Groups were compared with ANOVA ($p<0.05$). Pearson's correlation coefficient was used to test for associations between the two markers, p53 and p21^{WAF1}. Results: No leukoplakia recurred and no malignant transformation was observed whitin a follow-up period of 3-6 years. The mean percentage of p53 staining in the basal and parabasal layers was similar in all groups. p21^{WAF1} staining differed between layers was as follows: in the basal, only 3 to 4% of cells were stained, while in the parabasal, between 16 and 28% of the epithelial cells were stained in the four different studied groups with no statistically significant difference ($p>0.05$). Conclusions: Our findings failed to differentiate the non-dysplastic lesions by means of p53 and p21^{WAF1} immunostaining, notwithstanding similar profiles between non-dysplastic and dysplastic leukoplakias were observed.

Key words: Leukoplakia. Oral cancer. Tumor suppressor protein p53. Cyclin-cependent Kinase inhibitor p21.

INTRODUCTION

Leukoplakia is the most common potentially malignant disorder of the oral mucosa. The World Health Organization (WHO) defines it as "a white patch or plaque that cannot be characterized, clinically or pathologically, as any other disease". It is a diagnosis made by exclusion and to achieve the definitive histopathological diagnosis it must be complemented by incisional or excisional biopsy[9].

Epithelial dysplasia has always been considered one of the most important features concerning malignant transformation of oral leukoplakias, but, recent studies have shown a substantial amount (3.9 to 11%) of non-dysplastic leukoplakias undergoing malignant progression[7,8,20]. Additionally, these studies have failed on confirming the relationship between grade of dysplasia and risk of malignant transformation.

Notwithstanding malignant transformation may also take place in non-dysplastic leukoplakia there is no information available in the literature considering

differences about behavior or risk of malignant transformation associated with each of the most common non-dysplastic abnormalities. Most studies evaluating the risk of malignant transformation have restricted their focus to the presence or absence of epithelial dysplasia and considered non-dysplastic leukoplakias as one single group, regardless of the different epithelial disorders observed[13,24].

Non-dysplastic leukoplakias may present several microscopic epithelial alterations. Waldron and Shafer[28] (1975), after a microscopic analysis of 3,256 leukoplakias, found that 80.1% showed different matches of hyperorthokeratosis, hyperparakeratosis and acanthosis and 16.7% of the leukoplakias presented epithelial dysplasia.

Leukoplakias may be characterized by a range of disorders in epithelial cell proliferation and differentiation[19]. One possible way to analyze alterations in non-dysplastic leukoplakias is by the assessment of proteins related to cell cycle control. Proliferation and differentiation processes are intimately related to cell cycle regulation, and changes in the machinery that regulates cell cycle may trigger the transformation to malignant neoplasms[25].

The p53 protein, a product of the TP53 tumor suppressor gene, is expressed in the late G1 phase and arrests cell cycle progression to the S phase, to allow the repair of damaged DNA; if the damage persists or cannot be repaired, p53 will trigger cell death by apoptosis[18]. Because of its short half-life, p53 is found in low concentrations in epithelial cells. However, in potentially malignant disorders and oral cancer, an increase in its immunohistochemical labeling takes place, suggesting that changes in p53 expression may be a characteristic of initial stages of oral carcinogenesis[10,21].

In order to block the cell cycle, p53 induces transcription of the $p21^{WAF1}$ protein, which is encoded by the WAF-1 gene. The $p21^{WAF1}$ protein forms a quaternary complex with cyclin, cyclin-dependent kinase (CDK) and the proliferating cell nuclear antigen (PCNA), thereby bypassing phosphorylation of the pRb protein by the active cyclin-CDK complex and inhibiting progression of the cell cycle from G1 to phase S. Furthermore, when bound to the PCNA, $p21^{WAF1}$ directly inhibits DNA replication during phase S[5]. The immunohistochemical labeling of $p21^{WAF1}$ is also altered during the process of oral carcinogenesis, increasing in number and intensity as the severity of histological findings progresses. Therefore, it has been suggested that changes in $p21^{WAF1}$ expression may contribute to, or reflect, carcinogenesis in oral epithelium[3,22,30].

The aim of this study was to evaluate the immunolabeling profile of p53 and $p21^{WAF1}$ in non-dysplastic leukoplakias: acanthosis, hyperkeratosis and acanthosis combined with hyperkeratosis, and compare them with dysplastic leukoplakias.

MATERIAL AND METHODS

The sample comprised lesions with a clinical diagnosis of leukoplakia obtained from patients treated at the Oral Pathology Department of the School of Dentistry of the Federal University of Rio Grande do Sul, Brazil, during a period of 3 years. As inclusion criteria all patients in this study should present clinically a solitary and homogenous leukoplakia not exceeding 2 cm, in such a way that only excisional biopsies were performed to ensure that the histopathological diagnosis represented the true nature of the leukoplakia. Exclusion criteria that removed patients from the sample were lesions with identified different sources of traumatic factors (for example frictional chronic irritation, trauma from an ill-fitting denture or other appliance, oclusal trauma from an opposing tooth, masticatory trauma from eating hard foods)[2], presence of *Candida albicans* infection and presence of verrucous leukoplakia. The experiments were undertaken with the understanding and written consent of each subject and the patients remain under clinical follow-up.

All samples were fixed in 10% neutral buffered formalin. The biopsy fragments were sectioned longitudinally at their central portion, and, in the preparation of paraffin blocks, both fragments were positioned in such a way that the histological sections could show the whole extension of the epithelium in the center of the lesion. Sections were stained with HE and histopathological diagnoses were confirmed in accordance with the WHO classification for leukoplakias[1], taking into account the anatomical site peculiarities.

Two 3-µm sections were obtained from each paraffin block for immunohistochemical staining. Sections were deparaffinized in xylene, rehydrated in alcohol and immersed in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase. Antigen retrieval was performed for 20 min in a steamer (Arno, São Paulo, SP, Brazil) using a low-pH retrieval solution (DakoCytomation, Carpentaria, CA, USA). The slides were incubated at 25ºC for one hour with the monoclonal anti-p53 (DO-7, 1:50, DakoCytomation) and monoclonal anti-$p21^{WAF1}$ (SX118, 1:30, DakoCytomation) antibodies. The detection system used was EnVision+ (DakoCytomation). Sections were counterstained with haematoxylin and mounted with Entellan (Merck KGaA, Darmstadt, Hessen, Germany). Sections of amygdala and of oral squamous cell carcinoma were used as positive controls for $p21^{WAF1}$ and p53, respectively. Negative controls were provided by omission of the primary antibody in sections of the same lesions used as positive controls.

Images of five selected fields indicative of the epithelial disorder under investigation were captured at 200x magnification using the Qcapture software version 2.81 (Quantitative Imaging Corporation, Inc.; Surrey, BC, Canada) in addition to a binocular microscope CX41RF model (Olympus Latin America Inc., Miami, FL, USA) coupled to a camera Qcolor 5, Coolet, RTV (Olympus Latin America Inc.) and

Table 1- Clinicopathological characteristics of leukoplakias and patterns of p53 and $p21^{WAF1}$ immunostaining

Case	HD	Age	Gender	Location	$p21^{WAF1}$				p53			
					BL (+ cells %)	PL (+ cells %)	IL	SL	BL (+ cells %)	PL (+ cells %)	IL	SL
1	ED	65	M	Lower lip	4.4	4.4	+	-	38.4	38.8	+	
2	ED	41	M	Tongue	13.4	35.8	+	-	44.2	71	+	-
3	ED	29	F	Tongue	2.8	25.6	+	-	82.6	80.4	+	+
4	ED	57	F	Tongue	5.4	38	+	-	54.2	69.2	+	-
5	ED	59	F	Gingiva	3.8	34.8	+	-	13	36.6	+	-
6	ED	75	F	Tongue	1.8	24.6	+	-	66.2	85.2	+	-
7	ED	46	F	Gingiva	1	27.2	+	-	51.4	37	+	-
8	ED	77	M	Tongue	2	19.8	+	-	36.6	32.2	+	-
9	ED	44	M	Buccal mucosa	2.8	17	+	-	15.2	26	+	-
10	ED	47	M	Tongue	13.2	51.6	+	-	41.4	42.2	+	+
11	ED	55	M	Tongue	3.6	26.6	+	-	50.4	61.4	+	-
12	A	29	M	Tongue	0	7.6	-	-	42	42.4	+	-
13	A	31	F	Buccal mucosa	3.6	28.2	+	-	68.2	68.8	+	+
14	A	53	M	Lower lip	11.8	39.8	+	-	81.8	84.8	+	+
15	A	42	F	Gingiva	0.6	29	+	-	6.6	4.8	+	+
16	A	72	M	Buccal mucosa	5	18.2	+	-	42.6	51.6	+	-
17	A	47	F	Lower lip	5.8	36.4	+	-	16.6	31.6	+	+
18	H	67	F	Gingiva	4.4	17	+	+	55	76.4	+	+
19	H	71	F	Gingiva	0	1.6	+	+	51.6	55.4	+	+
20	H	28	M	Tongue	15.2	36.2	-	-	79.4	87.4	+	+
21	H	46	F	Gingiva	0.6	14.6	+	-	12.4	27.2	+	+
22	H	65	M	Gingiva	2.4	25	+	-	28	39.2	+	-
23	H	71	M	Gingiva	0	0.4	+	-	44.4	40.8	+	-
24	H	43	M	Tongue	13	15.4	+	+	71.6	68.6	+	-
25	H	41	M	Lower lip	2.4	24.8	+	-	74.4	75.4	+	+
26	H	44	F	Gingiva	0	12.6	-	-	12	11.6	+	-
27	AH	42	M	Buccal mucosa	2.8	30.2	+	-	36.6	36.8	+	-
28	AH	37	M	Palate	2	3.6	-	-	41.6	51.2	+	+
29	AH	62	F	Gingiva	5.4	4.8	+	-	34.2	22.6	+	+
30	AH	34	F	Buccal mucosa	4.2	38.2	+	+	78.6	68.6	+	+
31	AH	66	M	Gingiva	3.2	13.6	+	-	62.2	72.2	+	-
32	AH	50	M	Lower lip	4.2	22.6	+	-	89.6	89.4	+	+
33	AH	24	F	Buccal mucosa	3	16	+	-	61.4	57.4	+	+
34	AH	47	F	Lower lip	1.2	21.2	+	-	46.6	55.2	+	+
35	AH	44	M	Buccal mucosa	2.8	33	+	-	26.6	27	+	-
36	AH	54	F	Buccal mucosa	10.4	26	+	-	57.6	58	+	+

HD=Histopathological diagnosis; ED=Epithelial dysplasia; A=Acanthosis; H=Hyperkeratosis; AH=Acanthosis and Hyperkeratosis; M=Male; F=Female; BL=Basal Layer; PL=Parabasal Layer; IL=Intermediate Layer; SL=Superficial Layer

connected to a computer (Dimension 5150, Dell, Eldorado do Sul, RS, Brazil). Microscopic fields with subepithelial inflammatory infiltrate, overlapping cells, and artifact areas were excluded.

ImageTool for Windows version 3.0 (Health Science Center, University of Texas, San Antonio, TX, USA) was used to count 500 cells from the basal layer and 500 from the parabasal layer, thus determining the percentage of cells marked as positive. Cells were classified as positive when their nuclei were brown-stained. A qualitative analysis was also carried out to determine the presence or absence of immunohistochemical staining in the intermediate and superficial layers. Layers were defined as follows: the basal layer was defined as containing cells with at least one point of contact with the basement membrane; the parabasal layer was composed of cell layers immediately above the basal layer, the shape of the cells was not flattened; the superficial layer was characterized by the most superficial cells, presenting a typical flattened shape; and the intermediate layer was defined as containing epithelial cells between the parabasal layer and the superficial layer[11]. Analysis and quantification were performed by a single observer blinded to the group to which the lesions belonged (the fields had been coded).

Intraexaminer calibration was performed by means of a second analysis of one in every 10 fields observed and calculated via the intraclass correlation coefficient (ICC=0.996 for the basal layer and ICC=0.994 for the parabasal layer). For qualitative variables, the Kappa (k=1) test was used, showing no statistically significant differences between readings. The SPSS software for Windows version 11.0 was used to analyze the results. The statistical analyses met assumptions of normality and homogeneity of variance. Therefore, groups were compared with ANOVA considering a 5% significance level ($p<0.05$). Pearson's correlation coefficient was used to test for associations between the two markers, p53 and p21^{WAF1}. The study was independently reviewed and approved by the Research Ethics Committee at the School of Dentistry, Universidade Federal do Rio Grande do Sul (protocol no. 01/07).

RESULTS

The sample was comprised of 6 specimens of acanthosis, 9 of hyperkeratosis, 10 of acanthosis combined with hyperkeratosis and 11 of epithelial dysplasia. The mean age of patients was 50.13 (±14.51) years old, 19 were male and 17 female. The most common anatomic site of the leukoplakias was gingiva (n=11) followed by tongue (n=10), buccal mucosa (n=8), lip (=6) and palate (n=1). All clinicopathological characteristics of the sample are specified on Table 1. The patient's follow-up ranged from 3 to 6 years, and no recurrence, new leukoplakia lesion or progression to oral squamous cell carcinoma was observed.

All leukoplakia samples showed positive immunohistochemical staining results for the proteins p53 and p21^{WAF1} (Figure 1). The mean percentage of p53 staining in the basal and parabasal layers was similar, ranging from 42 to 53% in all four groups. On the other hand, p21^{WAF1} staining differed between layers: in the basal layer, only 3 to 4% of cells were stained, while in the parabasal layer, between 16 and 27% of the epithelial cells were stained in the four

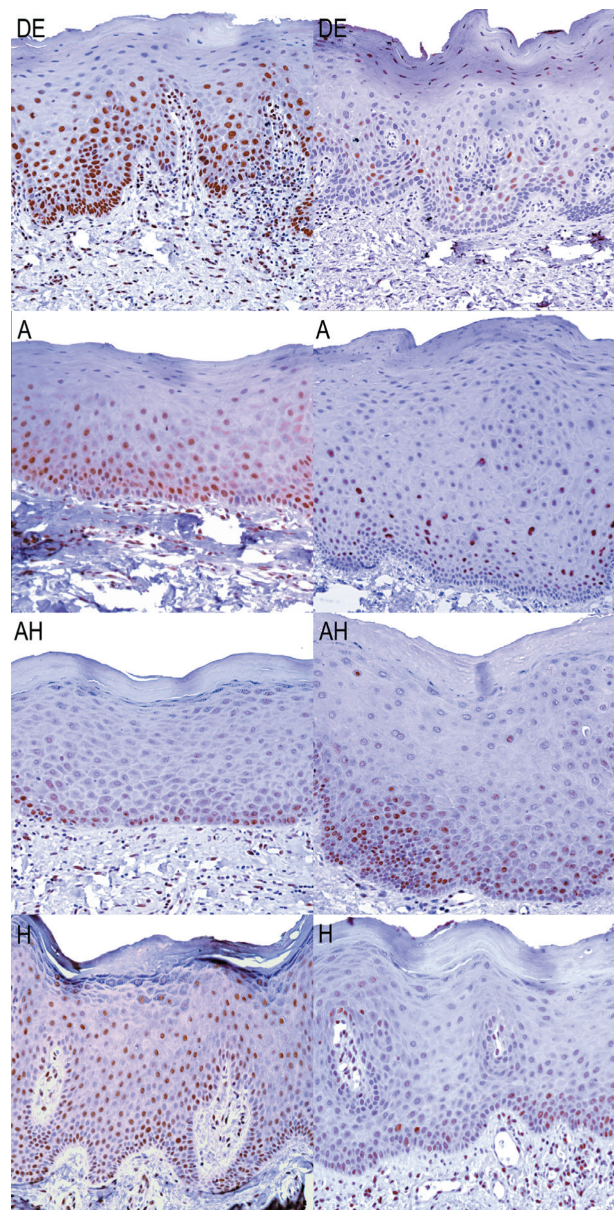

Figure 1- p53 and p21^{WAF1} expression in oral leukoplakias. Leukoplakias with: epithelial dysplasia (DE); acanthosis (A); acanthosis in combination with hyperkeratosis (AH); hyperkeratosis (H). p53 and p21^{WAF1} immunohistochemical expressions are shown in the left and right columns, respectively

Table 2- Distribution across groups of mean numbers and mean percentages of p53 positive cells with standard errors (±SE) for the basal and parabasal layers

Groups	Mean n±SE	Mean %±SE	p
BASAL LAYER			
Epithelial dysplasia	224.36±30.37	44.87±06.07	
Acanthosis	214.50±58.91	42.90±11.78	
Hyperkeratosis	238.22±42.81	47.64±08.56	0.79
Acanthosis + hyperkeratosis	267.50±31.88	53.50±06.38	
PARABASAL LAYER			
Epithelial dysplasia	263.64±31.80	52.73±06.36	
Acanthosis	236.67±57.47	47.33±11.49	
Hyperkeratosis	267.78±42.48	53.56±08.50	0.951
Acanthosis + hyperkeratosis	269.20±32.68	53.84±06.54	

Table 3- Distribution across groups of mean numbers and mean percentages of $p21^{WAF1}$ positive cells with standard errors (±SE) for the basal and parabasal layers

Groups	Mean n±SE	Mean %±SE	p
BASAL LAYER			
Epithelial dysplasia	24.64±6.51	4.93±1.30	
Acanthosis	22.33±8.73	4.47±1.75	
Hyperkeratosis	21.11±9.70	4.22±1.94	0.96
Acanthosis + hyperkeratosis	19.60±4.06	3.92±0.81	
PARABASAL LAYER			
Epithelial dysplasia	138.82±18.64	27.76±3.73	
Acanthosis	132.67±24.33	26.53±4.87	
Hyperkeratosis	82.00±18.92	16.40±3.78	0.165
Acanthosis + hyperkeratosis	104.60±18.23	20.92±3.65	

Table 4- Distribution of samples exhibiting positive p53 and $p21^{WAF1}$ staining in the intermediate and superficial layers

Groups	total (n)	p53		$p21^{WAF1}$	
		Intermediate +	Superficial +	Intermediate +	Superficial +
Epithelial Dysplasia	11	11 (100%)	3 (27%)	11 (100%)	0 (0%)
Acanthosis	6	6 (100%)	4 (66%)	5 (83%)	0 (0%)
Hyperkeratosis	9	9 (100%)	5 (55%)	7 (77%)	3 (33%)
Acanthosis + hyperkeratosis	10	10 (100%)	7 (70%)	9 (90%)	1 (10%)

different studied groups. No statistically significant differences (p>0.05) were observed between the groups for immunohistochemical staining for p53 and $p21^{WAF1}$ (Tables 2 and 3).

Table 4 lists the number of specimens with positive staining results in the intermediate and superficial layers. Pearson's correlation coefficient showed a weak but significant direct correlation between p53 and $p21^{WAF1}$ in basal layer cells (R=0.361), but not in the parabasal layer (R=0.250).

DISCUSSION

The results of the present study show that p53 and $p21^{WAF1}$ are overexpressed in oral leukoplakias, both dysplastic and non-dysplastic ones. One important finding is that dysplastic and non-dysplastic leukoplakias showed similar profiles of p53 and $p21^{WAF1}$ immunostaining. Oral leukoplakia has been extensively studied in the literature, and most studies have compared dysplastic with

the non-dysplastic leukoplakias classified into a single group. This study assessed p53 and p21WAF1 immunolabeling of the three most common non-dysplastic leukoplakias.

A significant number of leukoplakias in which epithelial dysplasia is not detected on biopsy may undergo malignant transformation[7,8,20]. Therefore, it is lacking in the literature information about differences in behavior profiles among the different histologic subtypes of non-dysplastic leukoplakias. Although no differences were observed in the present study, Hildebrand, et al.[6] (2010), analyzing cell proliferation rate through AgNOR staining, verified that between different subtypes of non-dysplastic leukoplakias only the acanthosis group showed a proliferative behavior similar to that found in epithelial dysplasia.

In all epithelial disorders assessed here, the immunohistochemical labeling of p53 was altered. Under physiological conditions, p53 expression should be restricted to a few cells in the epithelial basal layer[21]. However, a larger number of immunohistochemically stained cells was found in all layers of the samples without differences between the groups. In the present study, we did not grade dysplasia samples due to the difficulties involved in reaching a diagnostic consensus and to the subjectivity involved in grading epithelial dysplasia[17,29]. Additionally, epithelial dysplasia were classified as absent or present, mainly because our focus was to analyze the non-dysplastic leukoplakias and use dysplasia just as a comparison group.

Despite the large number of studies about p53 immunohistochemical labeling, a closer comparison with previous works is difficult. There is no standardization of staining methods, antibody clones, sensitivity of the detection system or quantification and interpretation of results[4]. Several studies have shown a correlation between immunohistochemical p53 labeling and mutation of the TP53 gene. However, under certain conditions, the wild type protein may be accumulated in cells as a response of different types of cellular stress, as result of the association between the wild type p53 and other proteins, or of the disruption of its degradation pathway, making it more easily detectable[15]. The clone DO-7 monoclonal antibody used in this study recognizes both proteins[16].

A possible explanation for the increased expression of p53 in this sample could be the presence of genetic damage, which may lead to oral cancer if progressively accumulated. Studies have shown that genetic alterations can occur in early stages of oral carcinogenesis, particularly at chromosome 3, in keratotic and non-dysplastic lesions[23]. However, we observed a positive correlation between p53 and p21WAF1 expression; usually, a p53-independent induction of p21WAF1 takes place in response to cell differentiation signals, while p53-dependent induction occurs in response to cellular stress[27]. The observed correlation contradicts other studies that did not detect such association and which have suggested that mechanisms independent of the action of p53 are involved in inducing p21WAF1 expression in potentially malignant lesions and oral cancer[14,26].

p21WAF1 staining was observed mainly in the parabasal layer of the epithelium, with isolated positive cells in the basal layer, which is in agreement with the results of other studies[3,22], and the rate of positive cells was also similar between the groups. There is evidence that as the epithelial abnormalities progress from dysplasia to squamous cell carcinoma, p21WAF1 labeling increases in number of positive cells, and from being restricted to the parabasal layer it extends to all epithelial layers[3,22]. While in normal epithelium p53 and p21WAF1 are detectable only in basal layer or in few isolated cells, in the parabasal layer it is observed that as the severity of the epithelial alteration increases the expression of these proteins seems to up rise through the epithelium. Most studies on p53 and p21WAF1 labeling discriminate just two layers, the basal and all other layers are assessed as a single suprabasal layer. In this study, we decided to analyze 4 different epithelium layers to have a more detailed visualization of this phenomenon.

The reason why p21WAF1 expression increases in association with oral carcinogenesis has not yet been elucidated. This increase could represent an attempt to control cell proliferation, which is possibly overcome by other factors which stimulate carcinogenesis and overload the inhibitory function of p21WAF1. Alternatively, the action held by p21WAF1 could be favoring cell proliferation[5,30], since the inhibitory function of p21WAF1 is stechiometrically regulated: many molecules of p21WAF1 are needed to inhibit a cyclin-CDK complex, whereas a single molecule may favor active cyclin-CDK complex binding[31].

Although surgical removal still seems to be the predominant method of treatment of oral leukoplakias, there remains little evidence that there is a reliable and safe method to prevent the recurrence of leukoplakias, and the potential for oral squamous cell carcinoma development[12], since no randomized controlled trials have been undertaken to test this hypothesis. We have opted for including only lesions smaller than 2 cm so that excisional biopsy could be performed because, even though there is no evidence of its efficacy, it allows us identifying the true nature of leukoplakia, as many studies have shown that incisional biopsies are not representative of the whole lesion[8].

In the present study, the inclusion criteria were established to standardize some aspects of the

sample, such as clinical aspect and lesion size, so we could focus on the histopathological finding and its significance for the leukoplakia behavior. So far no recurrence or transformation was observed.

CONCLUSION

The different types of non-dysplastic lesions showed similar profiles of p53 and p21^{WAF1} staining. The lack of significant differences between non-dysplastic leukoplakias and dysplastic leukoplakias is in agreement with recent data presented in the literature. There is no consensus on the most appropriate management for non-dysplastic leukoplakias. While this question is not answered, our findings suggest that all leucoplakias, independent of their histopathological diagnosis, must be considered as potentially malignant disorders and the patients should be kept under regular long-term clinical follow-up with a specialist.

ACKNOWLEDGEMENTS

This study was funded by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS; PROAP 04/2005; Process nº 0410882) and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We are especially grateful to Chris K. Danilevicz for image technical assistence. The authors declare that they have no conflict of interest.

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