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ORIGINAL ARTICLE

Bcl-2 expression and its possible influence on malignant transformation of oral lichen planus

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Summary

Purpose: *The aim of this study was to investigate the malignant potential of oral lichen planus (OLP) on the basis of the expression of the Bcl-2 marker in healthy individuals (H), patients with OLP and patients with squamous cell carcinoma (SCC), and to establish potential interdependence between expression of Bcl-2 and the different clinical and histopathological parameters in H, OLP, and SCC.*

Methods: *Immunohistochemistry for Bcl-2 was carried out using an avidin-biotin peroxidase complex method. Tissue sections were treated with mouse monoclonal antibody against Bcl-2 (124, DAKO A/S, Denmark; dilution 1/100). Immunohistochemical parameters measured included total tissue area, total stained area and intensity of stain.*

Results: *Keratinocytes were not Bcl-2 positive in H and were sparsely positive in OLP. The number of lymphocytes stained with Bcl-2 was significantly lower in H as compared to patients with SCC and OLP. Bcl-2 staining was weak to moderate in OLP, and moderate to intense in SCC. Bcl-2-positive lymphocytes were more expressed in older OLP patients (>55 years), and in OLP specimens with orthokeratinized epithelium, less developed acanthosis and highest grade of lymphocyte expression.*

Conclusion: *Bcl-2 may not serve as a prognostic biomarker in oral cancer development from OLP, but it could help in selecting patients with higher need of follow up to prevent malignancy.*

Key words: Bcl-2, oral lichen planus, premalignant lesion, squamous cell carcinoma

Introduction

OLP is a T-cell mediated chronic inflammatory oral mucosal disease of unknown etiology. Basal cells appear to be the target of T lymphocytes. The inflammatory cells and the keratinocytes express cytokines and display membrane receptors that together induce a signaling network which is thought to be crucial in the pathogenesis of OLP [1]. This network mainly consists of pro-apoptotic and anti-apoptotic signals. Apoptosis, as a form of cell death, is characterized by morphological as well as biochemical criteria and can be considered as a counterpart of mitosis. Better-understood genes implicated in the control of apoptosis are c-*myc*, Bcl-2 and p53.

Currently, it is recognized that Bcl-2 is a member of a group of genes with sequence homology known as the Bcl-2 gene family, which regulates cell death and survival. The members of Bcl-2 family can be subdivided into two groups, one with anti-apoptotic (Bcl-2, Bcl-X_L) and one with pro-apoptotic properties (Bcl-X_S, Bax). Overexpression of the anti-apoptotic proteins (Bcl-2, Bcl-X_L) can inhibit the activity of pro-apoptotic proteins (Bcl-X_S, Bax) [2]. The Bcl-2 proto-oncogene is a known inhibitor of apoptosis. Bcl-2 encodes an integral membrane protein that is localized on the mitochondrial outer membrane, nuclear envelope, plasma membrane and endoplasmatic reticulum. It is found in different lymphoproliferative diseases, in normal epithelia with active proliferative compartment and in various epithelial neoplasias [3]. Furthermore, its association with precancerous lesions suggests a role in the early stage of carcinogenesis [4].

Mounting evidence suggests that oral carcinogenesis is correlated with a progressive accumulation of genetic alterations in molecules that play cru-

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cial roles during apoptosis. The presence of genetic changes in precancerous lesions of oral mucosa underscores the significance of apoptotic deviations during the early steps of malignant transformation [5,6]. Overexpression of Bcl-2 results in an alteration of programmed cell death with persistence of cells that fail to die. Members of the Bcl-2 family, such as Bcl-2 and BAX, are differentially expressed in normal oral epithelium, epithelial dysplasia and SCC [5-7].

We hypothesized that immunohistochemical expression of the Bcl-2 protein in keratinocytes and lymphocytes could show progression from healthy oral tissue, through premalignant lesions (OLP), to oral SCC.

The aim of this study was to investigate the malignant potential of OLP, based on the expression of the Bcl-2 marker in keratinocytes and lymphocytes of H, OLP patients, and patients with SCC. We also wanted to see for any potential interdependence between the expression of Bcl-2 and expression of different clinical and histopathological parameters in H, OLP, and SCC.

Methods

The study included 40 patients with diagnosed OLP. Diagnosis of OLP was made on the basis of clinical findings and histopathological features on the material retrieved from the Laboratory of Pathobiology, University hospital in Nice, France, between February 2003 and May 2006.

Two control groups were included in this research. The first one consisted of 13 healthy persons (H) scheduled for oral surgery because of existing benign hyperplasia (fibroma, papilloma). Immunohistochemical examination was carried out on the oral mucosa without inflammatory changes and was the same as for patients with OLP. In the second control group, immunohistochemical examination was carried out on SCC of the oral mucosa obtained by biopsy from 12 patients with highly differentiated SCC. The immunohistochemical procedure was also the same as for patients with OLP.

Clinical examination

The following clinical parameters were considered in the study: sex, age, clinical type of disease, duration of disease, subjective symptoms, presence of other diseases and drugs used in the therapy of different pathological conditions. Qualitative analysis of oral changes and their localization was determined using clinical examinations (on the basis of size and structure of lesions). Written informed consent was obtained from all study subjects.

Histopathological studies

Histopathological analysis was performed on material obtained by biopsy of oral mucosa of patients with diagnosed OLP. A biopsy specimen from each patient was fixed in formalin, embedded in paraffin and routinely sectioned and stained with H&E or PAS.

The following parameters were analyzed on the epithelium: keratinization (presence and type of keratinization/ortho or parakeratinization), granular layer, hyperplasia, atrophy, acanthosis, hyperbasal cells (multiplication of basal cells, as their distribution in more than one layer of cells), liquefacient degeneration, necrotic cells (keratinocyte cell death), Civatte (eosinophilic) bodies, spongiosis (dilatation of intercellular spaces within the basal cell layer of epithelium), exocytosis (penetration of lymphocyte infiltrate from submucosa to the lower layers of epithelium). In the zone of the basal membrane and submucosa the following structures were analyzed: Max-Joseph spaces (focal separation of epithelium from connective tissue), thickening of the basal membrane, cellular infiltration, presence of lymphocytes, presence of Civatte (eosinophilic) bodies and edema. Semiquantitative examination was performed for all mentioned parameters. Their degrees of expression were also taken into consideration, and were described as mild, moderate and intense.

Immunohistochemical analysis

Immunohistochemistry was carried out using an avidin-biotin peroxidase complex method [8]. For the immunostaining method with antigen retrieval citrate buffer (pH 6.0) solution was used. Tissue sections were transferred to a beaker containing each buffer solution and incubated at 95º C in a microwave oven for 17 min to unmask the site of antigen. After taking away from the microwave oven, the tissue sections were left for 20 min in a beaker at room temperature. Then, they were rinsed with phosphate buffered saline (PBS) and incubated with 0.3% H_2O_2 for 15 min to block the endogenous peroxidase activity. The tissue sections were then incubated with normal goat serum for Bcl-2 staining and treated overnight at 4º C with a mouse monoclonal antibody against Bcl-2 (124, DAKO A/S, Denmark) - dilution 1/100. The samples were incubated with biotinylated animal-matched secondary antibodies (DAKO A/S, Denmark) at room temperature, and after rinsing with PBS, they were incubated again with the complex avidin-biotin peroxidase for 45 min. Protein expression was visualized using a kit (DAKO) developed with diaminobenzidine (DAB)-H_2O_2 substrate complex. Each section was left in the DAB solution up to 15 min and

counterstained lightly with Mayer's hematoxylin (Hemalun). PBS was used for all washings between the applications of the staining reagents and also as a diluent buffer for the antibodies.

Tonsilar tissue (nuclear, cytoplasmic) served as positive control for the Bcl-2 antibody. Staining was considered positive when the nuclear, cytoplasmic or basal membrane staining of the mucosal epithelium cells of OLP was compatible with that of positive control. For negative control, the same procedure was carried out with normal serum instead of each antibody. Immunohistochemical measurement parameters included total tissue area, total stained area and intensity of stain. Five hundred keratinocytes or lymphocytes were randomly counted in epithelium (basal and prickle cell layer) and submucosa. Semi-quantitative and semi-qualitative evaluation was performed for Bcl-2 staining according to the following criteria: quantitative (0 - negative, grade 1 [1-5%], grade 2 [5-25%], grade 3 [25-50%], grade 4 [50-100%]); and qualitative (0 - negative, 1 (+) weak, grade 2 (++) moderate, grade 3 (+++) intense).

Statistical analysis

The collected data were analysed using Fisher and Fisher exact test for pairs, to make comparison of differences between the examined groups. Comparative analysis between clinical, histopathological and immunohistochemical parameters were considered only in cases where statistical significance was reached or almost reached.

Results

The OLP group comprised 28 (70%) women and 12 (30%) men, with a mean age of 58.3 years (range 33 - 81). There were 4 (10%) patients with systemic lupus erythematosus (SLE), 7 (17.5%) with cardiac disease, one (2.5%) with hepatitis C; 15 (37.5%) patients were smokers and 11 (27.5%) consumed alcohol. Healthy controls included 7 (53.8%) women and 6 (46.2%) men, while the SCC group of patients included 8 (66.7%) women and 4 (33.3%) men. Erosive type of OLP was the most frequently found (35 patients - 70%), followed by reticular (11 patients - 22%), plaque-like (3 patients - 7.5%) and bullous (one patient - 2.5%). The results of the histopathologic study are shown in Tables 1 and 2.

Immunohistochemical analysis showed that keratinocytes were Bcl-2-negative in H and sparsely positive in OLP. Differences in the number of lymphocytes stained with Bcl-2 were statistically significant between H and both SCC and OLP groups ($p < 0.001$) (Table 3). There were more Bcl-2-stained cells in SCC specimens (Figure 1) in comparison to OLP (Figure 2), but without reaching statistical significance ($p=0.46$; Table 3). In most cases, SCC and OLP specimens exhibited 1-5% positively marked lymphocytes (50% of SCC patients and 42.5% of OLP patients).

Bcl-2 staining of lymphocytes was weak to moderate in OLP, and moderate to intense in SCC specimens (Table 4). In H specimens all lymphocytes were Bcl-2-negative. Comparing all 3 groups, statistically

Table 1. Histopathologic analysis of epithelium in patients with oral lichen planus (n = 40)

Variable	*Expression*	*n*	*%*
Keratosis	No	0	0
	Parakeratosis	20	50
	Orthokeratosis	20	50
Granulosis	No	12	30
	+	17	42.5
	++	6	15
	+++	5	12.5
Acanthosis	No	22	55
	+	10	25
	++	5	12.5
	+++	3	7.5
Hyperplasia	No	28	70
	+	8	20
	++	3	7.5
	+++	1	2.5
Atrophy	No	32	80
	+	6	15
	++	2	5
	+++	0	0
Hyperbasal cells	No	13	32.5
	+	21	52.5
	++	4	10
	+++	2	5
Liquefacient degeneration	No	0	0
	+	10	25
	++	13	32.5
	+++	17	42.5
Civatte bodies	No	1	2.5
	+	21	52.5
	++	15	37.5
	+++	3	7.5
Necrotic cells	No	1	2.5
	+	16	40
	++	22	55
	+++	1	2.5
Spongiosis	No	29	72.5
	+	9	22.5
	++	2	5
	+++	0	0
Exocytosis	No	1	2.5
	+	30	75
	++	7	17.5
	+++	2	5

Table 2. Histopathologic analyses of basal membrane zone and submucosa in patients with oral lichen planus (n = 40)

Variable	*Expression*	*n*	*%*
Max-Joseph spaces	No	39	97.5
	+	1	2.5
	++	0	0
	+++	0	0
Thickening of basal membrane	No	21	52.5
	+	16	40
	++	2	5
	+++	1	2.5
Cellular infiltration	No	0	0
	+	8	20
	++	11	27.5
	+++	21	52.5
Lymphocytes	No	0	0
	+	7	17.5
	++	33	82.5
Civatte bodies	No	5	12.5
	+	33	82.5
	++	2	5
Edema	No	30	75
	+	8	20
	++	2	5
	+++	0	0

significant difference in staining intensity of Bcl-2 protein was found between H and SCC and H and OLP ($p < 0.001$). Although lymphocytes were stained more intensely in SCC specimens, differences in staining intensity of Bcl-2 protein between SCC and OLP were insignificant (p=0.22).

Older OLP patients were Bcl-2-positive significantly more frequently than younger ones (p=0.01). Low percentage (level 1) of Bcl-2-positive lymphocytes in OLP specimens was seen mostly in grade 1 of acanthosis (ACA; 62.5%), while ≥2 grade of ACA was Bcl-2-negative in most cases (60%). Differences between the examined groups were insignificant (p=0.13). Low percentage (level 1) of Bcl-2-positive lymphocytes was discovered mostly in OLP specimens with grade 2 lymphocyte expression (45.81%). Specimens with grade 1 lymphocyte expression were in most cases Bcl-2-negative (80%). Nevertheless, the difference between these two groups was insignificant (p= 0.12).

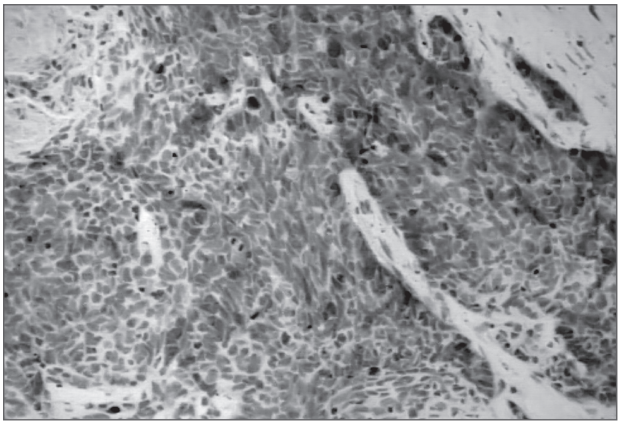

Figure 1. Localization of Bcl-2 antigen in squamous cell carcinoma specimen. Low percentage of lymphocytes intensively stained with Bcl-2 (immunohistochemical technique, × 200).

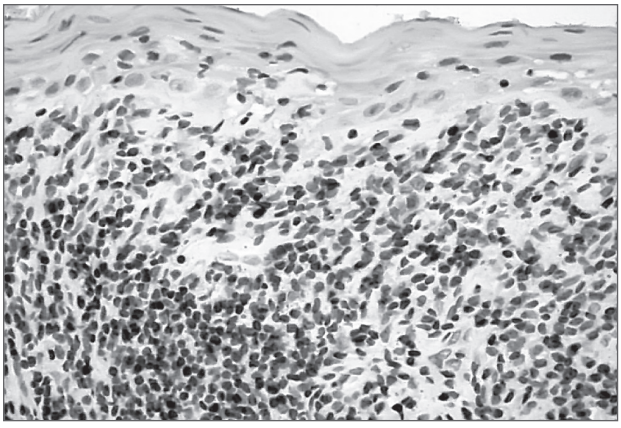

Figure 2. Localization of Bcl-2 antigen in oral lichen planus specimen. Low percentage of lymphocytes moderately stained with Bcl-2 (immunohistochemical technique, × 200).

Table 3. Comparison of Bcl-2 expression in lymphocytes between SCC, OLP and H

Variable	*SCC*		*OLP*		*H*	
	n	*%*	*n*	*%*	*n*	*%*
0 Bcl-2-negative	1	8.33	12	30	13	100
1 Bcl-2 (1–5%)	6	50	17	42.5	0	0
2 Bcl-2 (5–25%)	3	25	6	15	0	0
3+ Bcl-2 (25–50%)	2	16.67	5	12.5	0	0
Total	12	100	40	100	13	100

$p < 0.001$
Fisher test; Fisher exact test for pairs: SCC vs. LP, p = 0.46; SCC vs. H, $p < 0.001$; LP vs. H, $p < 0.001$
SCC: squamous cell carcinoma, OLP: oral lichen planus, H: healthy individuals

Table 4. Comparison of Bcl-2 stain intensity in lymphocytes between SCC, OLP and H

Variable	*SCC*		*OLP*		*H*	
	n	*%*	*n*	*%*	*n*	*%*
0 Bcl-2-negative	1	8.33	12	30	13	100
1 Bcl-2 grade 1	4	33.4	15	37.5	0	0
2 Bcl-2 grade 2^+	7	58.33	13	32.5	0	0
Total	12	100	40	100	13	100

$p < 0.001$
Fisher test; Fisher exact test for pairs: SCC vs. LP, p = 0.22; SCC vs. H, $p < 0.001$; LP vs. H, $p < 0.001$
SCC: squamous cell carcinoma, OLP: oral lichen planus, H: healthy individuals

Bcl-2 staining of lymphocytes was more intense in the older group of OLP patients in comparison to younger ones, with statistical significance (p=0.05). Moderate to high intensity of Bcl-2 staining (grade 2^+) of lymphocytes in OLP specimens was detected more often in orthokeratinization (45%) of the cases in comparison to parakeratinization (20% of the cases), but without statistical significance (p=0.20; Figure 3). Weak intensity of Bcl-2 staining (grade 1) of lymphocytes was identified more often in OLP specimens with grade 2^+ of ACA (62.5%, Figure 4). Moderate to high intensity of Bcl-2 staining (grade 2^+) was detected in low percentage in OLP specimens with developed ACA. Weak intensity of Bcl-2 staining (grade 1) of lymphocytes was identified more often in OLP specimens with grade 2 of lymphocyte expression (42.86%). Specimens with grade 1 of lymphocyte expression were in most cases Bcl-2-negative. The difference between the examined groups was statistically significant (p=0.01). No other significant relationships were found between the examined clinical or histopathological variables in OLP and Bcl-2 expression.

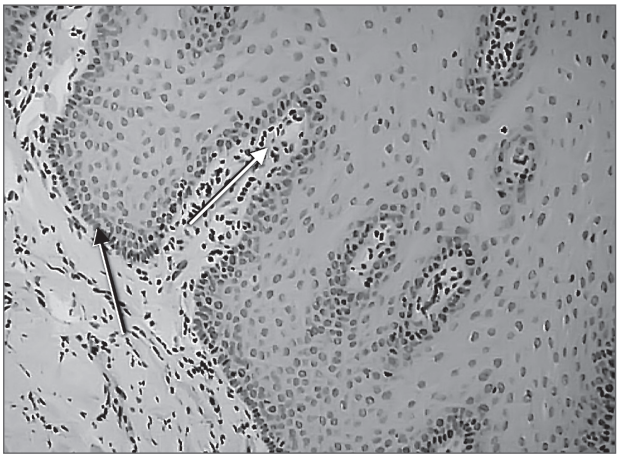

Figure 4. Weak intensity of Bcl-2 staining of lymphocytes (white arrow) in oral lichen planus specimen with high expression of acanthosis (black arrow). Immunohistochemical technique, ×200).

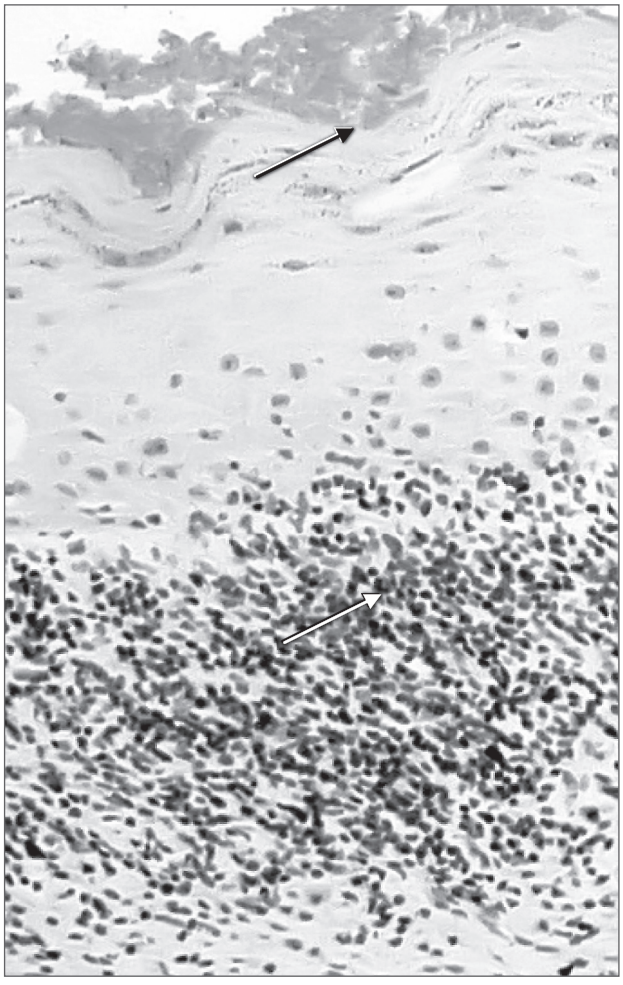

Figure 3. Moderate intensity of Bcl-2 staining of lymphocytes (white arrow) in oral lichen planus specimen with orthokeratinized epithelium (black arrow). Immunohistochemical technique, ×200.

Discussion

The general negative Bcl-2 staining of oral keratinocytes in normal oral mucosa and OLP suggests concomitant loss of other prosurvival molecules, increase in the proapoptotic molecules, or no role of Bcl-2 in the basal cell death that typifies OLP [9].

The number of lymphocytes stained with Bcl-2 in this survey was significantly lower in healthy individuals in comparison to patients with SCC and OLP. In most cases SCC and OLP specimens exhibited 1-5% Bcl-2-positively marked lymphocytes. We noticed progressive changes in the number of lymphocytes stained with Bcl-2 from normal tissue to OLP and SCC. Bloor and coworkers noticed that numerous lymphocytes in the superficial corium and those infiltrating the epithelium in OLP were positive for Bcl-2, indicating inhibition of apoptosis [7]. The low frequency of apoptosis may be explained by the need to preserve the epithelial structure, since massive apoptotic death would remove the basal cells responsible for epithelial regeneration [10]. It seems that Bcl-2 constitutes an important mechanism for the persistence of infiltrate in OLP [9,11]. This protein may contribute to the longevity of lymphocytes and macrophages in the interface infiltrate, and therefore to lesion chronicity in OLP [9]. Furthermore, numerous studies [11-14] demonstrated lymphocyte proliferation in the infiltrate, with lymphocyte expression of Ki-67 and cyclin-D [15]. The low rate of apoptosis and increased rate of proliferation in the epithelium may underlie a predisposition to cancer

in OLP. Although infrequent, OLP lesion can undergo malignant transformation [16-19].

In our study Bcl-2 staining of lymphocytes was weak to moderate in OLP, and moderate to intense in SCC specimens. In healthy controls all specimens were negative. The intensity of Bcl-2 staining changed gradually from normal mucosa to SCC. Generally, terminally differentiated (keratinizing) cells show diminished immunoreactivity for Bcl-2 [7]. Singh and coworkers established increased Bcl-2 expression in sequentially progressing epithelial dysplasia, but diminished expression in differentiated carcinomas. The Bcl-2 oncoprotein is topographically restricted to cells in proliferating zones and cells with long lifespans, and it is downregulated in terminally differentiated cells. These results suggest that altered Bcl-2 protein may be involved in early carcinogenesis. In our study, Bcl-2-positive lymphocytes were discovered mostly in OLP specimens with highest grade of lymphocyte expression. We support the hypothesis that Bcl-2 overexpression plays a crucial role in allowing the cell to escape apoptotic death and be left with long time of inflammation [20]. The chronic inflammatory infiltrate characterizing OLP could be the main cause of tissue malignancy, since the inflammatory cells and their cytokines are able to cause tumor development [21]. Some molecules and radicals generated by inflammatory cells can act as mutagenic agents for epithelial cells or influence important cell cycle regulation mechanisms, e.g. apoptosis, cell cycle arrest and cell proliferation, among others [22]. Balkwill and Mantovani have underlined the role of macrophages, dendritic cells, inflammatory cytokines and chemokines, which are able to cause DNA damage, bypass p53 tumor suppression function, and influence growth, survival, angiogenesis and invasion [23]. The above account of cell responses suggests an epithelium at high risk for malignant transformation. This transformation is much less frequent in OLP than might be expected because of the control exerted by p53, which is activated in a high percentage of epithelial cells and preferentially arrests the cell cycle for DNA repair [11]. However, a high price may be paid for increased cell proliferation in an epithelium under intense aggression, which may underlie the malignant transformation capacity of OLP [24].

According to our results, Bcl-2-positive lymphocytes were more frequently expressed in older OLP patients (>55 years), and in OLP specimens with orthokeratinized epithelium, less developed ACA and highest grade of lymphocyte expression. Numerous authors found that an oral cancer most frequently develops in older group of OLP patients (between the 6th and 7th decade of life) [25]. In leukoplakia, where orthokeratinization is extremely expressed, both the lymphocytes and suprabasal keratinocytes are Bcl-2-positive in higher percentage than in OLP [26]. Papa et al. discovered positive influence of the inflammatory mediator PGE_2 on the intensity of Bcl-2 coloration [27]. Bcl-2 staining was more intense with highest degree of lymphocyte expression, probably due to the development of intensive inflammatory process. The question is: are the older OLP patients with the above-mentioned histopathological changes more predisposed to cancer? We are not sure about that, but we think that those clinical and histopathological variables could serve as a sign for more rigorous follow up of patients. Of course it is possible that the results from these comparative analyses are just accidental. Further studies are needed to confirm or deny these relationships.

Generally, Bcl-2 was more expressed in lymphocytes of OLP patients compared with healthy persons, but less compared with patients with SCC. Nevertheless, Bcl-2 may not serve as a prognostic biomarker in oral cancer development from OLP, but it could help select those patients with higher need of follow up to prevent malignancy.

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