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ORIGINAL ARTICLE

Ki-67 expression in oral lichen planus

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Summary

Purpose: *The monoclonal antibody Ki-67 detects a nuclear antigen that is present only in proliferating cells. This is of particular interest for the analysis of the proliferation rates of malignant tumors. The aim of this study was to investigate the malignant potential of oral lichen planus (OLP) on the basis of expression of Ki-67 in healthy individuals (HI), patients with OLP and patients with squamous cell carcinoma (SCC), and to see for any potential interdependence between Ki-67 expression and different clinical and histopathological parameters in OLP.*

Methods: *Immunohistochemistry for Ki-67 was carried out using an avidin-biotin peroxidase complex method.*

Results: *Ki-67 was more expressed in keratinocytes and lymphocytes of OLP patients compared with HI, but less compared with patients with SCC. Keratinocytes and lymphocytes stained with Ki-67 in OLP patients were significantly higher in males, and in OLP specimens showed less developed civatte bodies (CB) and thickening of the basal membrane (TBM).*

Conclusion: *Ki-67 may not serve as prognostic biomarker in oral cancer development from the initially diagnosed OLP, but it could help selecting patients with higher need of follow up for prevention of malignancy.*

Key words: Ki-67, oral lichen planus, premalignant lesion, squamous cell carcinoma

Introduction

OLP is a T-cell-mediated chronic inflammation of oral mucosa 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 consists mainly of pro-apoptotic and anti-apoptotic signals [2]. 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 studied genes implicated in the control of apoptosis are c-*myc*, *Bcl-2* and *p53* [3].

The Ki-67 monoclonal antibody detects a nuclear antigen that is present only in proliferating cells. Ki-67 nuclear antigen is present in G_1, S, G_2 and M phase of the cell cycle, but is absent in G_0 cells, so the antigen disappears when proliferating cells are induced to differentiate into resting cells. Its expression increases with cell cycle progression and reaches its peak during G_2 and M phase [4]. With the help of Ki-67, a simple and rapid determination of the growth fraction of a given cell subset has become possible. This is of particular interest for the analysis of the proliferation rates of malignant tumors [5].

Mounting evidence suggests that oral carcinogenesis is correlated with a progressive accumulation of genetic alterations in molecules that play crucial 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 [4,5]. Ki-67 is differentially expressed in normal oral epithelium, epithelial dysplasia and SCC [6-8].

We hypothesised that immunohistochemical expression of Ki-67 in keratinocytes and lymphocytes could show progression from healthy oral tissue, through premalignant lesions (OLP), to oral SCC.

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The aim of this study was to investigate the malignant potential of OLP based on the expression of Ki-67 in keratinocytes and lymphocytes of OLP patients, HI, and patients with SCC. We also investigated to establish any potential interdependence between expression of Ki-67 and different clinical and histopathological parameters in OLP patients.

Methods

The study included 40 patients with diagnosed OLP. Diagnosis of OLP was made on the basis of clinical analysis 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 formed in this research. The first one consisted of 13 HI, and immunohistochemical examination was carried out on the oral mucosa without inflammatory changes. The biopsies of the oral tissue were obtained from individuals with benign lesions, already indicated for oral surgical treatment. Immunohistochemical procedure was the same as in 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 in patients with OLP.

Clinical examination

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

Histopathological examination

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

The following epithelial parameters were analysed: keratinization (presence and type of keratinization/ortho or parakeratinization), granular layer, hyperplasia, atrophy, acanthosis (ACA), hyperbasal cells (HBC; multiplication of basal cells), liquefaction degeneration, civatte (eosinophilic) bodies (CB), necrotic cells (dead keratinocytes, spongiosis), dilatation of intercellular spaces within the basal cell layer of epithelium, exocytosis (penetration of lymphocyte infiltrates from submucosa to the lower layers of epithelium). In the zone of basal membrane and submucosa the following structures were analysed: Max-Joseph spaces (focal separation of epithelium from connective tissue), TBM, cellular infiltration (CI), presence of lymphocytes, presence of CB and edema.

Semiquantitative examination was performed for all mentioned parameters. Their degrees of expression were also taken into consideration during histopathological analysis, and they were described according to the following categories: mild, moderate and intense.

Immunohistochemical analysis for Ki-67

Immunohistochemistry was carried out using an avidin-biotin peroxidase complex method [9]. For immunostaining with antigen retrieval, modified citrate buffer solution was used. Tissue sections were transferred to a beaker containing 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 PBS and incubated with 0.3% H_2O_2 for 15 min to make a blockage of the endogenous peroxidase. The tissue sections were then incubated with normal goat serum for Ki-67 staining. They were treated overnight at 4º C with an already dissolved mouse monoclonal antibody against Ki-67 (MIB-1, DAKO A/S, Denmark). 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 avidin-biotin peroxidase for 45 min. Protein expression was visualized using a kit (DAKO) 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) was the positive control for the Ki-67 antibody. Staining was considered positive when the nuclear 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. The positive expression of Ki-67 (MIB-1) was evaluated by counting the number of positive cells per 1000 cells.

Statistical analysis

Collected data were analysed using Fisher exact test for pairs and Wilcoxon rank sum test with continuity correction, to make comparisons of differences among the examined groups. Comparisons of multilevel factors between groups were statistically analysed using Kruskal-Wallis rank sum test.

Interdependence between immunohistochemical and clinical or histopathological parameters were considered only in cases where statistical significance was reached or almost reached.

Results

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

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

Variable		*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
Liquefaction 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		*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

Table 3. Number of keratinocytes stained with Ki-67 in patients with squamous cell carcinoma, oral lichen planus and healthy controls

	Ki-67 Keratinocytes		
	SCC patients	*OLP patients*	*Healthy controls*
Mean±SD	145.9±97.6	100.9±75.9	60.9±72.7
Median	146	83	37
Range	5-278	3-267	0-223

SCC: squamous cell carcinoma, OLP: oral lichen planus, SD: standard deviation
Kruskal-Wallis rank sum test, p = 0.04; Fisher's Exact test for pairs: SCC vs. OLP, p = 0.17; SCC vs. healthy controls, p = 0.03; OLP vs. healthy controls, p = 0.06

The staining with Ki-67 was confined to the nucleus of keratinocytes and was homogeneous in most cases. Ki-67 labeling index was gradually increasing with progressive epithelial transformation: from normal (60.9 ± 72.7) through OLP (100.9 ± 75.9), to reach maximum in SCC (145 ± 97.6). Differences between those groups were statistically significant (p=0.04; Table 3). Significant difference was also found between SCC vs. HI (p=0.03).

Keratinocytes and lymphocytes were Ki-67 positive in significantly higher percentage in male OLP patients (Tables 4,5). Ki-67 labeling index increased with progression of HBC expression. The highest value of Ki-67 was at HBC grade 2+ (167 ± 95.37). Almost significant difference was found in this comparative analysis (p=0.07; Table 4). There were more lymphocytes stained with Ki-67 in OLP specimens - CB grade 1 in comparison to OLP specimens - CB grade 2+ (p=0.07; Table 5). Similar results were obtained in a case of TBM. There were more lymphocytes stained with Ki-67 in OLP specimens - TBM grade 1 in comparison to OLP specimens - TBM grade 2 (p=0.15; Table 5).

Discussion

Numerous studies [10-13] have demonstrated lymphocyte proliferation in the lymphocytic infiltrate, with lymphocyte expression of Ki-67 and cyclin-D [14]. The low rate of apoptosis and increased rate of proliferation

Table 4. Ki-67 expression in keratinocytes of oral lichen planus patients in different gender and different degrees of HBC

	Ki-67 Keratinocytes				
	*Gender**		*HBC***		
	Female	*Male*	*No*	*Grade 1*	*Grade 2+*
Mean±SD	79.75±71.39	143.2±69.17	66.75±51.84	101.4±71.22	167.7±95.37
Median	56	146.5	66.5	101	187
Range	3-230	25-267	5-186	9-213	3-267

SD: standard deviation, HBC: hyperbasal cells
*Wilcoxon rank sum test with continuity correction, W = 73, p = 0.017; **Kruskal-Wallis rank sum test, p=0.07

Table 5. Ki-67 expression in lymphocytes of oral lichen planus patients according to gender and different degrees of civatte bodies and thickening of basal membrane

	Ki-67 Lymphocytes						
	*Gender**		*CB***		*TBM****		
	Female	*Male*	*Grade 1*	*Grade 2+*	*No*	*Grade 1*	*Grade 2+*
Mean±SD	14.48±9.14	26.91±16.42	23.28±14.77	12.94±8.8	19.54±12.44	20.18±13.84	10.17±9.83
Median	13	21	20.5	12	17	19	5.5
Range	2-33	8-56	7-56	2-33	5-46	4-56	2-25

CB: civatte bodies, TBM: thickening of basal membrane, SD: standard deviation
*Wilcoxon rank sum test with continuity correction, W = 73.5, p = 0.029; **Kruskal-Wallis rank sum test, p = 0.07; ***Kruskal-Wallis rank sum test, p = 0.15

in the epithelium may underlie a predisposition to cancer in OLP. Although infrequent, OLP can undergo malignant transformation [15-18].

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 [19]. 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 [20]. 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, influence growth, survival, angiogenesis and invasion [21]. The above account of cell responses suggests an epithelium at high risk of 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 [22]. 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 [23].

In this study, Ki-67 labeling index of keratinocytes was gradually increasing with progressive epithelial transformation from normal, through OLP, to reach maximum in SCC. Similar results were obtained by Piatteli et al. with highest values for Ki-67 labeling index in severe dysplasia and carcinoma *in situ*. They found significant differences in the proliferation rates between normal tissue, premalignant lesions, dysplasia and carcinoma [24]. A recent study from Gonzales-Moles and coworkers also showed that Ki-67 was expressed more frequently in all tissue locations in the OLP group in comparison to the control healthy group [25]. OLP could develop a secondary proliferative condition, probably due to repeated breakdown of the cycling cells, leading to a heightened state of proliferation. The finding of a significantly higher state of cell proliferation in OLP compared with the normal oral mucosa raises the question whether this feature could be an important early step in some cases of OLP, that eventually transform to malignancy [23]. Proliferating cells have several mechanisms for repairing damaged DNA to reduce the number of genetic alterations. Overexpression of the wild-type p53 may be one such cell cycle control mechanism. Apart from p53, other molecules such as pRB, p21 and p27 could be important in cell cycle regulation in conditions of expressed cell proliferation [26,27]. The percentage of malignant transformation in OLP is probably low, partially because those mechanisms exist.

Keratinocytes and lymphocytes stained with Ki-67 in OLP patients from this survey were significantly higher in males. Taniguchi also found higher proliferation index in males, but without statistical significance [12]. Higher incidence of Ki-67 in the group of male OLP patients may be accidental, but it also could reflect higher malignant transformation capacity of OLP in males [12]. According to our results Ki-67 labeling index was higher with progression of HBC expression. Staining with Ki-67 protein was also expressed in cells of the parabasal layer, in areas where basal cells had un-

dergone liquefaction. Damage in the zone of basal cells is a signal of defense, resulting in basal and parabasal cell multiplication. Taniguchi et al. got similar results, with Ki-67 overexpression not only in the zone of flat epithelium but also in rete ridges with saw tooth appearance, suggesting budding of proliferative cells in these parts of basal cell layer [12]. The situation with CB was different, meaning that the number of keratinocytes and lymphocytes stained with Ki-67 was higher in OLP specimens with lower degree of expression of CB. CB represent the same kind of apoptotic products, a defense mechanism against uncontrolled mitosis. Apoptosis and mitosis are two opposite processes, and it is expectable that the proliferation rate of cells will be lower in a condition of up-regulated apoptosis, and *vice versa*.

Correlation between Ki-67 labeling index of lymphocytes and TBM was also negative. Increased thickening of basal membrane was developed more frequently in case of lower expression of Ki-67 protein on the membrane of lymphocytes. TBM is compensatory to the defects of the basal cell layer and the basal membrane itself, and develops in later disease stages. With basal cell liquefaction and basal membrane disintegration many lymphocytes infiltrate the zone of basal membrane. At early stages, when there are less lymphocytes in the submucosa, some of them could possibly reduplicate. As immunologic and inflammatory reactions get more serious, a need for further increase in the number of lymphocytes gets lower. Probably that's why the Ki-67 labeling index decreases with more expressed TBM.

The question is: are the male OLP patients with highest grade of HBC and the lowest grade of CB and TBM, more predisposed to cancer? We are not sure about that, but we think that these 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 this relationship.

Ki-67 was more expressed in keratinocytes and lymphocytes of OLP patients in comparison to HI, but less in comparison to patients with SCC. Ki-67 could be a useful parameter in selecting the patients with higher need of follow up to prevent malignancy.

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