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ORIGINAL ARTICLE

Role of tumor suppressor protein p16 in patients with oral lichen planus

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Summary

Purpose: *Oral lichen planus (OLP), is considered premalignant condition. This study tried to investigate the possibility of malignant transformation in OLP patients, by measuring the level of tumor suppressor protein $p16^{INK4A}$, which is a product of the cyclin-dependent kinase inhibitor 2 (CDK_2). CDK inhibitors may be responsible for maintenance of cell cycle. An imbalance in the cell cycle regulatory pathway involving p16/pRb may lead to unrestricted proliferation and tumorigenesis.*

Methods: *40 patients with OLP underwent biopsy. Two control groups were included in this research, 13 healthy persons and 12 patients with oral squamous cell carcinoma (OSCC). All tissue samples were treated immunohistochemically using avidin-biotin peroxidase complex method.*

Results: *number of patients with p16 positive cells was significantly higher in OLP (72.5%) in comparison to OSCC (33.33%). Although the prevalence of patients with p16 positive cells in OSCC was low, they expressed the highest percentage of p16 positive keratinocytes. Staining intensity of cytoplasm was also higher in p16 positive keratinocytes of OSCC in comparison to OLP.*

Conclusions: *These results suggest that inactivation of p16 is an early detectable event in oral tumorigenesis. P16 expression could also be connected with tumor grading and may be useful marker in oral tumor progression.*

Key words: *immunohistochemistry, malignant transformation, oral lichen planus, p16*

Introduction

Oral lichen planus (OLP) is a chronic inflammatory disease of unknown etiology which is classified as potentially malignant disorder. It is T-cell mediated disease and both antigen specific and nonspecific mechanisms, are assumed to be included in the pathogenesis of OLP [1,2].

Immunohistochemistry is a technique used for identifying cellular or tissue constituents (antigens) through antigen-antibody interactions. This technique is often used to distinguish the infiltrating cell population, describe the expression of molecules regulating apoptosis and clarify the mitotic activity of basal and parabasal cells (Ki-67, p16, cyclins) [3,4]. Overexpression of these molecules and intensive proliferative activity of basal and infiltrating cells are often present in carcinomas. So, based on the results of immunohistochemical examinations, malignant potential of OLP could be anticipated. Numerous potential markers have been identified and their connection with early detection, progression and prognosis of oral squamous cell carcinoma (OSCC) have also been discussed [2]. The most likely range of malignant transformation of OLP described in the literature varies between 0.1-3% [4,5].

Cell cycle is managed by the action of cyclin-dependent kinases (CDKs) and their main inhibitors p16, p21 and p27, which are important tumor suppressors [6]. $p16^{INK4A}$, a protein product of the CDK inhibitor 2 (CDKN2) gene, binds to and in-

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hibits CDK4 and/or CDK6, whose activity is critical in regulation of normal cell cycle progression [7,8]. Unlike the p21 family of cyclin-dependent kinase inhibitors, p16 specifically inhibits cyclin D-dependent kinases *in vitro*. This protein reduces phosphorylation of retinoblastoma protein (pRb) indirectly, and therefore G1 cycle arrest [8]. In normal cells the p16 protein is synthesized at a very low level. An imbalance in the cell cycle regulatory pathway involving p16-pRb may lead to unrestricted proliferation and tumorigenesis [9]. Rb is therefore an important tumor suppressor gene, and its inactivation in human tumors, through the inhibition of p16, has a positive impact on nuclear reprogramming, increasing both the number of reprogrammed cells and the kinetics of de-differentiation in proliferating cells [8,10]. P16 expression is also regulated by Rb: phosphorylation of Rb results in increased p16 expression which inhibits CDK4/6 resulting in increased levels of hypophosphorylated Rb which leads to decreased p16 expression [8]. Although there is a feedback loop between p16 and Rb, it has been shown that the level of p16 expression does not change significantly during cell cycle to correlate with the activation status of Rb [11].

As mentioned, p16 protein is involved in the antitumor response, promotes tumor suppression, and acts on the cell cycle [8]. Increased levels of p16 have been identified in senescent cells, therefore, it has been suggested that p16 may be responsible for the induction of cell senescence, preventing malignant cell transformation. Loss of p16 expression is a feature commonly found in neoplasms and has been detected early in the process of carcinogenesis.

The purpose of the study was to investigate the malignant potential of OLP on the basis of p16 expression. Examined was also the correlation of p16 with clinical and histopathological features in OLP.

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 Department of Periodontology and Oral Medicine in Belgrade, Serbia. The study protocol was approved by the Ethical Board of the School of Dental Medicine University of Belgrade. All participants provided written informed consent.

Two control groups were included in this research. The first one consisted of 13 healthy persons and immunohistochemical examination was carried out on the oral mucosa without inflammatory changes. The biopsies of the oral tissue were obtained from patients already indicated for oral surgical treatment. Immunohistochemistry was the same as in the 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. Immunohistochemistry was also the same as in 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 for the therapy of different pathological conditions. Qualitative analysis of oral changes and their localization was determined using clinical examination (size and structure of lesions).

Histopathological examination was performed as explained in the original article of Hadzi-Mihailović et al [4].

Immunohistochemical analysis for p16

Immunohistochemistry was carried out using the avidin-biotin-peroxidase complex method. For the immunostaining antigen retrieval, citrate buffer solution (pH 6.0) was used. Tissue sections were transferred to a beaker containing buffer solution and incubated at 95°C in a microwave oven for 17 min to unmask the site of antigen. After taken 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. Then, the tissue sections were incubated with normal goat serum for p16 staining. They were treated overnight at 4°C with a mouse monoclonal antibody against p16 (E_6H_4, DAKO A/S Denmark) – dilution 1/25. 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), 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.

Cervical cancer (nuclear, cytoplasmic) was the positive control for the p16 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. Five hundred keratinocytes or lymphocytes were randomly counted in epithelium (basal and prickle cell layer) and submucosa. Semi-quantitative and semi-qualitative evaluations were performed for p16 staining according to the following criteria: quantitative (0

Table 1. Percentage of keratinocytes stained with p16 in patients with squamous cell carcinoma, oral lichen planus and healthy controls

	OSCC *n (%)*	*OLP* *n (%)*	*H* *n (%)*
p16 negative	8 (66.67)	11 (27.50)	13 (100)
p16 ≤1%	0 (0)	12 (30)	0 (0)
p16 (1-5%)	0 (0)	16 (40)	0 (0)
p16 (5-10%)	4 (33.33)	1 (2.50)	0 (0)
Total	12 (100)	40 (100)	13 (100)

OSCC: oral squamous cell carcinoma, OLP: oral lichen planus, H: healthy controls, Fisher's test, p<0.001; Fisher's Exact Test for pairs: OSCC vs. OLP, p<0.001, OSCC vs. H, p = 0.03, OLP vs. healthy controls, p<0.001

- negative, grade1 [<1%], grade 2 [1-5%], grade 3 [5-10%], grade 4 [10-25%]); and qualitative (0 - negative, 1 (+) weak, grade 2 (++) moderate, and grade 3 (+++) intense).

Statistics

The data collected were analysed using Fisher exact test for pairs and Wilcoxon rank sum test with continuity correction, to make comparison of differences between the examined groups. Comparisons of multilevel factors between groups were statistically analyzed 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. P<0.05 was considered as statistically significant.

Results

The group of patients with OLP included 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. Fifteen (37.5%) patients were smokers and 11 (27.5%) consumed alcohol. There were 7 (53.8%) women and 6 (46.2%) men in the group of healthy individuals, while the group of patients with SCC included 8 (66.7%) women and 4 (33.3%) men. The erosive type of OLP was found in 35 patients (70%), followed by reticular in 11 patients (22%), plaque-like in 3 patients (7.5%) and bullous form in 1 patient (2.5%).

Immunohistochemical analysis showed that 40% of OLP lesions and 33.33% of OSCC lesions displayed both nuclear and cytoplasmic staining, while 32.5% of OLP lesions showed only cytoplasmic staining. P16 focal and diffuse patterns of positivity were found in OLP (72.5%) and OSCC (33.33%) specimens.

In most cases of OLP (70%), low to moderate percentage of cells (level 1 and 2) were p16 positive (Figure 1). The number of patients with highest percentage of keratinocytes stained with p16

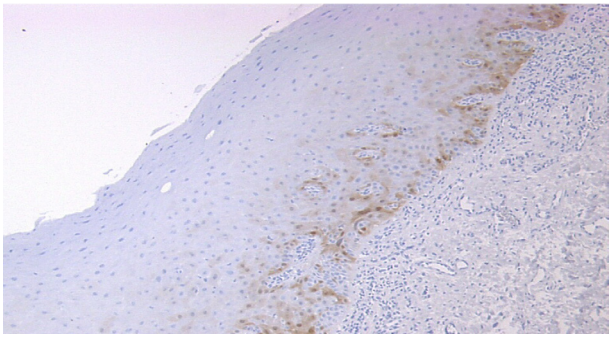

Figure 1. Staining of keratinocytes with p16 protein (patients with OLP, H&E ×40).

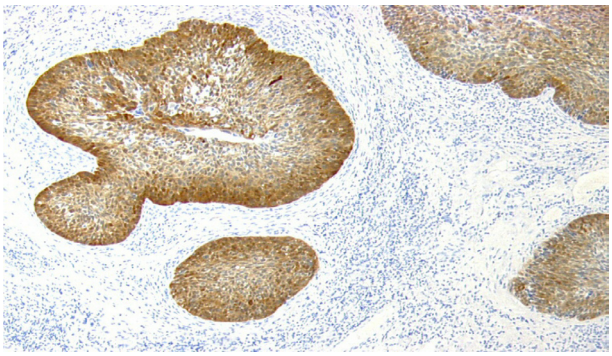

Figure 2. Staining of keratinocytes with p16 protein (patients with OSCC, H&E ×20).

(level 3) was higher by OSCC (33.33%, Figure 2) in comparison to OLP patients (2.5%). Statistical difference between these two groups was significant (p<0.001, Table 1). Nuclear and cytoplasmic colorations with p16 protein (Figure 3) were both expressed in 40% of OLP and 33.33% of OSCC specimens. All healthy controls (H) were negative on p16.

P16 staining was weak to moderate in the cytoplasm of 70% of OLP and moderate (33.33%) in the cytoplasm of OSCC specimens. Statistically significant difference between these two groups was established (p=0.02, Table 2). In the nucleus of OLP (27.5%) and OSCC (33.33%) specimens the staining

Table 2. Staining intensity of keratinocytes stained with p16 in patients with squamous cell carcinoma, oral lichen planus and healthy controls

	OSCC *n (%)*	*OLP* *n (%)*	*H* *n (%)*
p16 negative	8 (66.67)	12 (30)	13 (100)
p16 grade 1	0 (0)	12 (30)	0 (0)
p16 grade 2+	4 (33.33)	16 (40)	0 (0)
Total	12 (100)	40 (100)	13 (100)

OSCC: oral squamous cell carcinoma, OLP: oral lichen planus, H: healthy controls, Fisher's test, p<0.001, Fisher's Exact Test for pairs: OSCC vs. OLP, p=0.02, OSCC vs. H, p=0.03, OLP vs. healthy controls, p<0.001

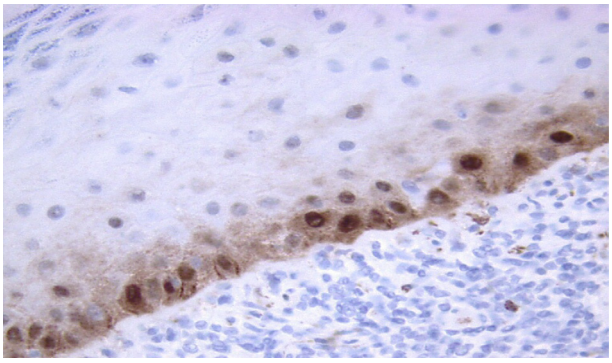

Figure 3. Staining of nucleus and cytoplasm of keratinocytes with p16 protein (patients with OLP, H&E ×40).

with p16 marker was mostly moderate. There was no statistically significant difference between these two groups (p=0.37).

Low and medium percentage (level 1 and 2) of p16 positive cells in OLP specimens, was predominately detected in females (78.57%), but statistical difference between male and females was not reached (p=0.12). P16 staining - grade 1 and 2+, was identified in most cases in female patients (78.57%). Statistical difference between sexes was insignificant (p=0.22).

Low percentage (level 1) of p16 positive cells in OLP specimens was discovered mostly by continual granular tissue (GT) (47.06%), and medium one (level 2) by focal GT (63.64%). Nevertheless, difference between these two groups was insignificant (p=0.19).

Weak intensity of p16 staining (grade 1) was identified more often in OLP specimens with 2nd+ grade of LD (36.67%), while moderate to high intensity of p16 staining (grade 2+) was detected equally (40%) in both degrees of LD. Statistical difference in this comparative analysis was not reached (p=0.15).

Low percentage (level 1) of p16 positive cells in OLP specimens was discovered mostly by CB grade 2+ (44.44%), and medium one (level 2) by CB grade 1 (47.62%). Nevertheless, the difference between these two groups was insignificant (p=0.23).

Weak intensity of p16 staining (grade 1) was identified more often in OLP specimens with 1st grade of CB (50%), while moderate to high intensity of p16 staining (grade 2+) was detected mostly by 2nd grade of CB (47.62%). Statistical difference was almost reached (p=0.08).

Weak intensity of p16 staining (grade 1) was identified in most cases of OLP specimens with 1st (28.57%) grade of CI. Moderate to high intensity of p16 staining (grade 2+) was detected more in OLP specimens with 2nd + grade of CI (33.33%). Statistical significance was not reached (p=0.21).

Negative correlation was established in OLP specimens between 1st grade of LY expression and intensity of p16 staining. Moderate to high intensity of p16 staining (grade 2+) was detected mostly by 2nd grade of LY expression (31.43%). Statistical difference between examined groups was insignificant (p=0.21).

Discussion

Montebugnoli et al [12] analysed the role of p16 in the progression of OLP into OSCC. They found an increased expression of p16 in 64% of OLP patients compared to only 28% of patients with oral leukoplakia. No differences were observed between samples from patients with OLP and those with nonspecific reactive inflammation. Interestingly, differences were observed between leukoplakia with signs of inflammation, where p16 expression was increased, and leukoplakia without signs of inflammation, where p16 expression was normal. These findings were in line with other studies that have shown a link between proinflammatory cytokines such as TNF-*a* and an increased p16 expression [13]. Moreover, an increased expression of p16 has been identified in 15 to 30% of cases of OSCC [14,15]. Goel et al [16] had revealed an increased expression of cytoplasmic p16 and CDK4 in OLP patients compared to that in normal mucosa. However, compared to OSCC, cytoplasmic expression of p16 and CDK4 were lower in OLP.

Cytoplasmic expression of p16 and CDK4 might be a predictor of the OLP malignant progression.

In the results of several studies all normal mucosal epithelia showed low percentage and weak positive staining of p16 protein [17,18]. All normal tissue samples from our research were, on the contrary, p16 negative. This difference is probably due to more severe criteria used in our study for establishing p16 positive cells, or in the use of different p16 antibodies.

Our results show that the number of patients with p16 positive cells was higher in OLP (72.5%) in comparison to OSCC (33.33%). Similar results got Shintani et al [17], with 71.4% of positive p16 cells in severe dysplasia and 31% of positive p16 cells in OSCC. These results suggest that inactivation of p16 is an early detectable event in oral tumorigenesis and premalignant oral lesions. In OSCC inactivation of p16 is even more expressed. Reed et al [19] reported over 80% of oral squamous cell carcinomas with completely lack of $p16^{INK4A}$ expression. Gradual alteration of p16 protein might contribute importantly to the multistep nature of oral carcinogenesis [17]. In the study of Purwaningsih et al [7] p16 expression was found in connective tissues as well as in suprabasal and basal layer of the oral squamous epithelium in almost 100% of patients with OSCC. It was found that p16 expression increased in OSCC compared with oral potentially malignant disorders.

The number of patients, from our study, with highest percentage of keratinocytes stained with p16 (level 3), was higher in OSCC (33.33%) in comparison to OLP (2.5%). Although the prevalence of patients with p16 positive cells in OSCC was low, all of them expressed the highest percentage of p16 positive keratinocytes. Besides, according to our results, staining intensity of cytoplasm was also higher in p16 positive keratinocytes of OSCC in comparison to OLP, while nuclear staining did not show significant difference between these two groups. Similar results got Bova et al [20] in their research. The marked overexpression of p16 in some OSCC specimens from this survey raises the possibility that some tumor cells, or tumors in general, are more subjected to the p16 expression than others. Indeed, Natarajan et al [21] have found complete absence of p16 positive cells in deeply invasive OSCCs, even in the epithelial-stromal interface, while at the same time they found p16 expression in many cells at the superficial circumferential margins of advanced OSCCs. Such p16 positive cells may either be premalignant cells of the field which the adjacent OSCC had arisen, or normal keratinocytes admixed with invasive OSCC. P16 expression could also be connected with tumor grading and it may be used as a helpful marker in oral tumor progression [22,23]. Some correlations between p16 protein and analysed clinical and pathological parameters in OLP specimens from this study were established, but statistical significance was not reached.

The p16 expression was detected in the majority of OSCC cases. These findings suggest that inactivation of p16 is an early detectable event in oral tumorigenesis and premalignant oral lesions. Intensity of p16 coloration was higher in OSCC patients in comparison to patients with OLP, although the prevalence of p16 positive keratinocytes was higher in OLP in comparison to OSCC.

Conflict of interests

The authors declare no conflict of interests.

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