IMMUNOHISTOCHEMICAL ANALYSIS OF ORAL MUCOSA LEUKOPLAKIA

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Abstract

In recent years, substantial changes have occurred in the structure of oral mucosa diseases, in particular an increased ratio of precancerous diseases, so that an effective non-invasive detection of any sign of malignancy appears as an urgent and most actual task of dentistry.

Aim: To study the proliferative activity of epithelial cells in Ki-67 antigenin patients with leukoplakia of the oral mucosa.

Materials and method: A complex clinical and laboratory examination was performed on 155 patients with oral leukoplakia, who addressed the Operative Dentistry Department of the "A.A.Bogomolets" National Medical University of Kiev between 2010 and 2014. All patients have been subjected to a careful clinical examination, which included: dental anamnesis, visual inspection, oral examination and digital palpation of oral mucosa and tongue mucosa, biopsy of leukoplakia lesions for cytological and histological examination.

Results: Histological evaluation of the material has been performed according to the WHO (2005) classification of leukoplakia. 10 (14%) sites of unaltered mucosa, 10 (14%) samples of hyperkeratosis without atypia, 14 (19%) biopsy specimens of hyperkeratosis SIN1, 15 (21%) – hyperkeratosis SIN2, 10 (14%) - SIN3 and 13 (18%) cases of squamous cell carcinoma were evidenced. Immunohistochemical investigation evidenced the presence of protein Ki-67 in the nuclei of epithelial cells. In the unmodified epithelium of the oral mucosa, all epithelial cells with stained nuclei are virtually located in the basal layer.

Conclusion: Against the general increase of the proliferative activity of epithelial cells with increasing SIN, a characteristic distribution of proliferating cells in the thickness of the epithelium was revealed for each studied group, as follows: in the control group and in leukoplakia without atypia, immunopositive cells are located in the basal layer, in leukoplakia (SIN1, SIN2 and SIN3) – in parabasal position while, in squamous cell carcinoma of oral mucosa, the distribution was uniform.

Keywords: *leukoplakia, oral mucosa, immunohistochemical evaluation.*

1. INTRODUCTION

A timely and effective diagnostic of the oral mucosa neoplastic lesions remains a serious challenge of dentistry. Annually registered in the world are nearly 400,000 cases of malignant tumors in the oral cavity, Ukraine recording more than 4,000 new cases [1].

Apparently, an effective clinical diagnostic of oral cavity malignant tumors based on tracing of precancerous diseases should not cause major difficulties, since the oral cavity is easily accessible for examination and palpation. However, in spite of this, 40-90% of patients begin the treatment in late stages (III - IV) of the disease and only about 20% - in early (I - II) stages. Accordingly, a 5-year survival of patients with cancer of the oral mucosa (stage I and II) by applying radiation, surgical and combined methods of treatment is attained in 65-85% of cases, while the ratio for stage III patients is of only 11-40%. Therefore, the results depend on an early detection of tumors, a timely diagnostic and treatment of precancerous diseases. The most frequent diagnostic errors occur in this stage of tumor development [1].

In recent years, substantial changes occurred in the structure of oral mucosa diseases, in particular an increased ratio of precancerous diseases, so that an effective, non-invasive detection of any sign of malignancy appears as an urgent and most actual task of dentistry.

Despite the apparent simplicity of clinical imaging of the predicted changes in the oral mucosa due to external localization, identification of cancer nosology, based exclusively on

examination and palpation, often leads to diagnostic errors, because the varying degrees of keratinization or ulceration, and even the marker elements of lesion are difficult to differentiate [2]. Especially difficult is an early diagnostic of malignancy, because its clinical symptoms appear a bit later than malignant cells transformation [2].

The percentage of incurable forms of oral mucosa cancer due to diagnostic errors reaches 58.4-70%, as stated by different authors. The situation is complicated by unresolved problems of precancer clinical diagnostic, especially the difficulty of differentiating the beginning of malignancy, and the choice of precancerous treatment. There are neither absolute nor relative indications for biopsy, especially in the stages of conservative treatment. The literature offers no example of diagnostic criteria at clinical level, to which the dentist might refer for choosing the most adequate method of precancerous disease treatment.

Most authors discuss the difficulties of the auxilliary methods of diagnostic (cytology, cheilostomatoscopy, biomicroscopy, optical coherence tomography) [2]. These methods are quite subjective, as they merely describe the qualitative changes produced in the affected tissues. One of the quantitative estimation methods of the morphological changes is determination of the immunohistochemical markers [3].

Implementation of modern methods of morphological verification of cancer in everyday practice has greatly improved the quality of diagnostics and treatment of malignant neoplasms. The methods of modern molecular oncology have extended, so that immunohistochemical researches can determine not only the histological diagnosis of the tumor, but also its morphofunctional characteristics from the viewpoint of aggressiveness and prognosis of precancerous lesions of the already developed cancer [2,3].

Proliferation markers also have a highly informative diagnostic value in predicting the development of malignant tumors [4-6]. The proliferative activity is a leading factor in the mechanism of malignant transformation of cells and biological behavior of the already present tumors. The perspective marker of proliferative activity is an antigen Ki-67 expression of virtually all phases of the cell cycle and, accordingly, it expresses the magnitude of the proliferative pool [7-9, 10]. The gene encoding the Ki-67, located on the long arm 10 of chromosome Ki-67, refers to regulatory proteins. Its appearance coincides with the entry of cells into mitosis, so that it can be used as a universal marker of proliferation for evaluating the growth of malignant tumors [11,12].

Aim of investigation: to study the proliferative activity of epithelial cells on the expression of Ki-67 antigen in patients with leukoplakia of the oral mucosa.

2. MATERIALS AND METHOD

A complex clinical and laboratory examination was performed on 155 patients – 87 women (56.1%) and 68 men (43.9%) - with oral leukoplakia, who addressed the Operative Dentistry Department of the "A.A.Bogomolets" National Medical University between 2010 and 2014. The age of patients ranged from 25 to 70 years (average age 46.6 ± 2.63 years). Various forms of leukoplakia: smooth, elevated, verrucous, erosive and ulcerative were identified in these subjects.

All patients have been subjected to careful clinical examination, which included the following stages:

Dental anamnesis. Particular attention was paid to the identification of the characteristic symptoms of leukoplakia and of the factors contributing to its development, such as: chronic mechanical trauma of the oral mucosa (caused by irregular denture, sharp edge of the tooth, etc.), bad habits (smoking, alcohol consumption, holding of pencils, pens, nails, etc. in the mouth), contact with industrial harmful substances (alkalis, acids in the form of vapors and aerosols, other chemicals), chronic stress, chronic gastrointestinal diseases, etc. Patients with alcohol consumption and smoking habits have been reported in a simplified way, as "consume",

"do not consume" or "unknown". Also recorded was the duration of the bad habit: up to 1 year, 5 years, or more.

Visual inspection. Examination of skin and lip red zone; palpation of the regional lymph nodes; study of the functions of the temporo-mandibular joint, masticatory muscles, branches of the trigeminal and facial nerves.

Oral examination and digital palpation of oral mucosa and tongue mucosa.

Biopsy of leukoplakia lesion for cytological and histological examination. The biopsy material was fixed in 10% neutral formalin (pH 7.4). After holding it on the tissue processor, it was embedded into paraffin with a melting temperature of 54 °C, in view of histological and immunohistochemical (IHC) investigations. Serial sections (5 micrometers thick) were placed on glass slides coated with poly-L-lysine. Identification of tissue antigens was carried out by mouse monoclonal antibodies to K8 (TS1, "Thermoscientific") and Ki-67 (MM1,"Thermoscientific"). Detection of the immune complexes was carried out with an UltraVisionQuantoDetection System HRP ("Thermoscientific") system, the sections being dyed with Mayer's hematoxylin. The reaction with K8 was evaluated from the intensity of cytoplasmic staining, by the H-score criterion: 0 - no cytoplasmic staining; 1 - slight staining; 2 - moderate cytoplasm staining; 3 - intense cytoplasm staining. The Ki-67 proliferation index (IPKi-67) is determined by the number of immunoreactive cell nuclei to the total number of nuclei. These markers were detected in hundreds of cells of the four epithelium layers. Evaluation of these indices is performed in appropriate tissue areas. Cell counts were carried out at a magnification of 400.

Statistical processing was carried out by Statistica 6.0. standard methods. Taking into account the abnormal distribution of the statistical indices, a comparison of two independent groups was carried out by the non-parametric U-testMann-Whitney method. The differences in the average level of statistical significance at $p<0.01$ were considered reliable. The relationship between the severity of displastic changes of keratinocytes (without atypia, SIN1, SIN2, SIN3, squamous cell carcinoma) and the level of Ki-67 proliferation was studied with the Spearman correlation coefficient.

3. RESULTS

The complex clinical and laboratory examination of the 155 patients with a clinical diagnosis of oral leukoplakia led to the following results: 52 patients (33.6%) suffered from a mild form of the disease, 40 (25.8%) – serious form, 43 (27.7%) –verrucous and 20 (12.9%) - erosive and ulcerative forms of leukoplakia.

Histological evaluation of the material has been performed according to the WHO (2005) classification of leukoplakia. 10 (14%) sites of unaltered mucosa, 10 (14%) samples of hyperkeratosis without atypia, 14 (19%) biopsy specimens of SIN1 hyperkeratosis, 15 (21%) – SIN2 hyperkeratosis, 10 (14%) - SIN3 and 13 (18%) cases of squamous cell carcinoma were evidenced.

Squamous hyperplasia was characterized by an increased number of cells in the basal and spinous layers. No sign of cellular and tissue atypia was revealed (Fig. 1).

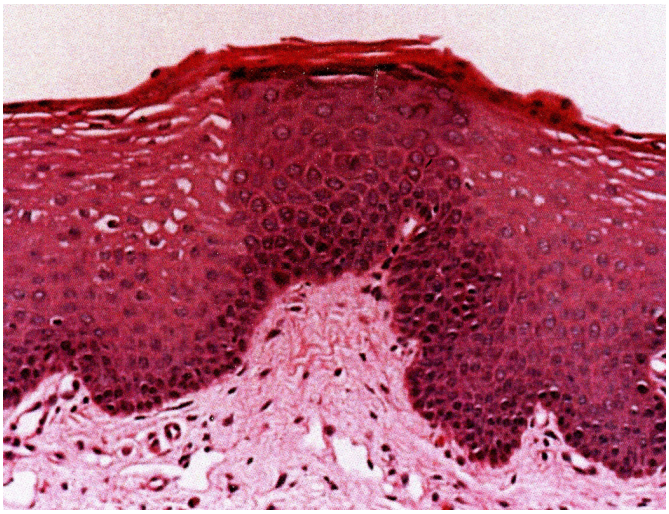

Fig. 1. Histological image of squamous hyperplasia

SIN1 leukoplakia was characterized by an intense proliferation of keratinocytes, with symptoms of minor cellular atypia and nuclear

polymorphism in the lower third of the epithelium (Fig. 2).

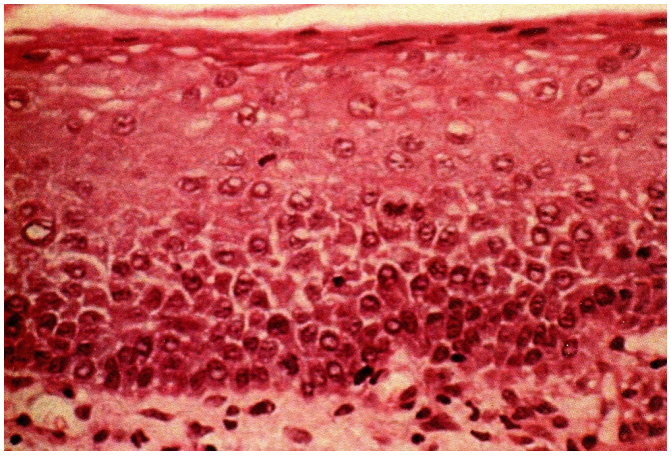

Fig. 2. Histological image of SIN1 leukoplakia

Characteristic to SIN2 leukoplakia was the location of the pathological process not only in the lower, but also in the middle third of the epithelial layer. Cellular and nuclear polymorphism was more pronounced, and hyperchromatism was observed, along with a higher nuclear-cytoplasmic coefficient and amount of mitosis in the basal layer. In some cases, changes in tissular architectonics, appearing as epidermal "loop-shaped" outgrowths, hyperkeratosis, were revealed (Fig. 3).

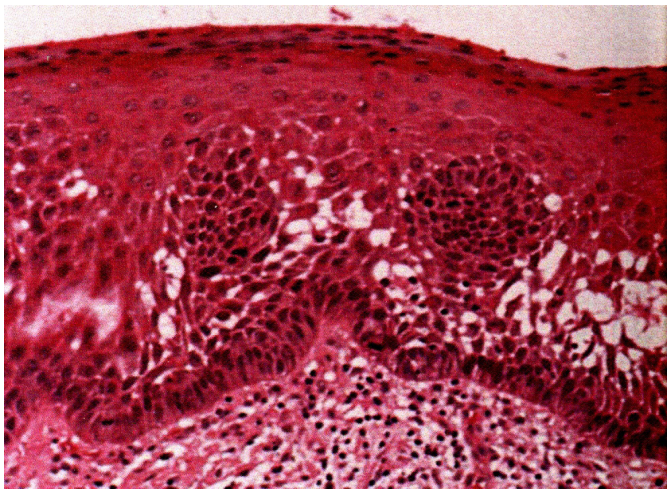

Fig. 3. Histological image of SIN2 leukoplakia

The SIN3 leukoplakia characteristic was the change of more than 2/3 of the epithelium height, expressed to a greater extent by all these signs of displasia, and by the increased number of nucleoli in cells with nuclear hyperplasia. Cells polymorphism was determined, and sometimes parakeratosis with foci of erosion was observed. Inflammatory phenomena, accompanied by a dense infiltration of lymphocytes with an admixture of plasma cells, were intensified (Fig. 4).

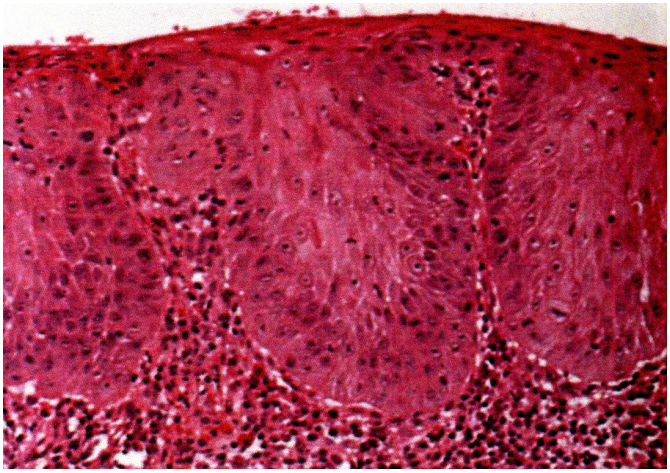

Fig. 4. Histological image of SIN3 leukoplakia

Histologic investigation of squamous cell carcinoma of the oral mucosa revealed disorderly arranged complexes of typical squamous cells with invasive growth in the deep lying layers of the submucosa. Manifested in varying degrees, characteristic for cellular atypia was the change in cell size and shape of nuclei, nuclear-cytoplasmic ratio, and the presence of polyploid forms of pathological mitosis (Fig. 5).

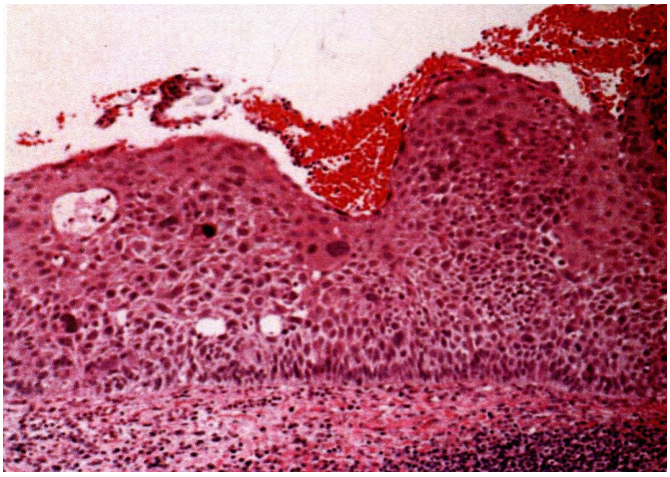

Fig. 5. Histological image of squamous cell carcinoma of oral mucosa

Immunohistochemical investigations revealed the expression of protein Ki-67 in the nuclei of epithelial cells. In the unmodified epithelium of

the oral mucosa, virtually all epithelial cells with stained nuclei are located in the basal layer. When oral leukoplakia with symptoms of SIN was present, the number of proliferating cells increased (Fig. 6).

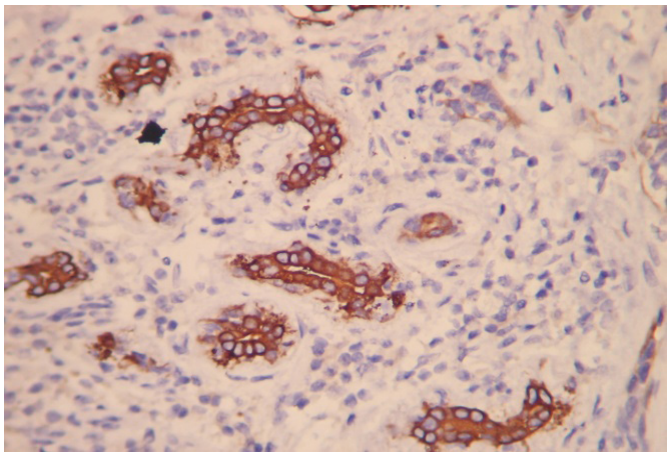

Fig. 6. Expression of protein Ki-67 in oral leukoplakia with SIN symptoms

Expression of the Ki-67 protein was observed in both basal and parabasal layers cells. The maximum number of immunopositive cells was localized in the second and third layers of the epithelial cells. The number of proliferating cells was significantly increased in squamous cell carcinoma. Figure 7 illustrates the immunohistochemistry reaction with antibodies to Ki-67 protein in the squamous cell carcinoma of the oral mucosa. A significant increase in the expression of the protein with epithelial cells, and nuclear staining of the most part of neoplastic cells in all four investigated zones were observed. Cells expressing the Ki-67 protein were distributed uniformly in the thickness of the epithelium.

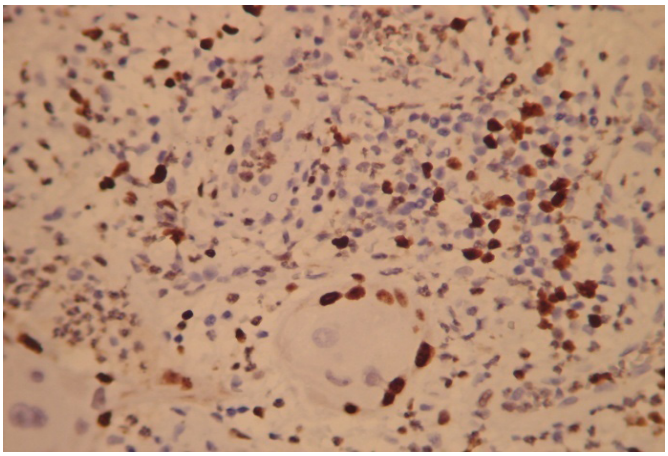

Fig. 7. Immunohistochemical reaction with antibodies to Ki-67 protein in the squamous cell carcinoma of the oral mucosa

The proliferative activity of the epithelium cells of oral mucosa was evaluated with the proliferation index (IP Ki-67), which represents the ratio of the number of cells with stained nuclei to the total number of cells. IP Ki-67 was determined in each cell layer of the unchanged epithelium, in leukoplakia (without atypia, SIN1, SIN2, SIN3) and squamous cell carcinoma of oral mucosa (Table 1).

Table 1. Layer-by-layer proliferation index of Ki-67 in normal epithelium, with leukoplakia (without atypia, SIN1, SIN2 and SIN3) and squamous cell carcinoma

Group	Diagnosis	1 layer/ zoneM±SD, %	2 layer/zone M±SD,%	3 layer/ zoneM±SD, %	4 layer/ zone M±SD,%
1	Normal epithelium, п=10	24.3±3.5	4.7±1.6	0	0 ^
2	Leukoplakia without atypia, n= 10	24.9±3.0	5.6±0.9	0	0
3	Leukoplakia SINl, n=14	22.4±3.5	6.9±1.3	4.1±1,6	0
4	Leukoplakia SIN2,n=15	25.4±3.0	16.6±1.6*	13.3±1,3*	0
5	Leukoplakia SIN3, n=10	12.7±2.3*	26.2±2.8*	24.5±3.0*	10.0±3.0*
6	Squamous cell carcinoma, n=13	75.3±12.2*	63.7±9.2'	69.1±12.5*	50.8±13.Г

*Significant differences between groups 1, 2, 3-4, p <0.01
Significant differences between groups 1, 2, 3, 4-5, p <0.01
Significant differences between groups 1, 2, 3, 4, 5-6, p <0.01

The results were also assessed by the index distribution of proliferating cells (IP Ki-67). In the unchanged layered squamous epithelium, protein expression Ki-67 was observed only in the 1st and 2nd layers of cells, a significant number of proliferating epithelial cells being localized in the basal layer, which represents 84% of all proliferating cells. In the 3rd and 4th cell layers, no proliferation was observed.

Evaluation of the proliferative activity of cells in various types of leukoplakia according to the 2005 WHO classification showed that, in the leukoplakia area without atypia, the distribution of stained cell nuclei was the same as in the unchanged oral mucosa epithelium: in the 1st and 2nd layers of cells, with predominant localization of proliferating cells in the basal layer, where IPKi-67 was 82%, and with no expression of the Ki-67 protein in the 3rd and 4th cell layers. In the case of SIN1 leukoplakia, a positive reaction was observed in the first three layers of cells. The bulk of proliferating cells was found in the basal layer, IP Ki-67 was 67%, and 33% - in parabasal position. In the 4th cell layer, the expression of protein Ki-67 was absent. With SIN2 leukoplakia, proliferation of epithelial cells was observed in the 1st, 2nd and 3rd layers, similarly with SIN1 leukoplakia, however the distribution of stained nuclei in the basal and parabasal layers was of 46% and 54%, respectively. The expression of protein Ki-67 in the 4th cell layer is not present.

With SIN3leukoplakia, the immunohistochemical reaction with antibody to Ki-67 was detected in all 4 cell layers, where the highest number of stained nuclei was observed in the parabasal layer: IRKi-67-69%. Distribution of stained nuclei in the 1st and 4th layers of cells was of 17% and 14%, respectively.

With squamous cell carcinoma of the oral mucosa, the proliferating epithelial cells were distributed fairly evenly from the basal membrane to the middle of the thickness of epithelium. In assessing the results by IPKi-67, also evidenced was the uniform distribution of proliferating epithelial cells. In the first zone, IP Ki-67 was equal to 29%, in the second - 25%, in the third - 27%, and in the fourth - 19%.

4. CONCLUSIONS

Against the general increase of the proliferative activity of epithelial cells with increasing SIN, for each studied group, a characteristic distribution of proliferating cells in the thickness of the epithelium was revealed: in the control group and in leukoplakia without atypia, the immunopositive cells are located in the basal layer, in leukoplakia (SIN1, SIN2 and SIN3) –in parabasal position, whereas, in squamous cell carcinoma of the oral mucosa, the distribution was uniform. A characteristic feature of SIN3 leukoplakia, comparatively with SIN1-SIN2leukoplakia, is a lower percentage of proliferating cells in the basal layer, compared with the parabasal one, which allows to establish a differential diagnostics between the types of leukoplakia and squamous cell carcinoma of oral mucosa. For determining the degree of epithelium neoplasia by an early diagnostics of the malignant transformation of cells, the distribution of immunopositive cells in the basal and parabasal layers should be considered.

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