

THE ROLE OF HUMAN PAPILLOMA VIRUS IN THE DEVELOPMENT OF ORAL LEUKOPLAKIA

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Abstract

Aim. To detect the Ki-67 proteins, P16INK4a and antigens of human papillomavirus of high oncogenic risk (HPV16) at hyperplasia, dysplasia and squamous cell carcinoma of the oral mucosa. **Materials and methods.** The biopsy material of the oral mucosa of 82 patients with leukoplakia was studied, including 42 women and 40 men. The average age of patients was 62 year. At carrying out histological examination unmodified epithelia, which were taken from the adjacent to leukoplakia areas of mucous membrane, were used as control. As a result of study, 10 (12%) areas of the unmodified mucous membrane, 35 (43%) biopsy materials without atypia leukoplakia, 16 (20%) biopsy materials of leukoplakia with dysplasia of varying degrees from SIN 1 to SIN 3 were studied; a squamous cell carcinoma was detected at 21 (25%) patients. **Results and discussion.** As a result of study, 10 (12%) areas of the unmodified mucous membrane, 35 (43%) biopsy materials without atypia leukoplakia, 16 (20%) biopsy materials of leukoplakia with dysplasia of varying degrees from SIN 1 to SIN 3 were studied; a squamous cell carcinoma was detected at 21 (25%) patients. IHC study of oral mucosa epithelium has revealed features of protein expression that characterizes the proliferative processes and viral antigens during different morphological manifestations of leukoplakia showing human papilloma virus replication in proliferating cells of stratified squamous epithelium. **Conclusions.** At various morphological variants of leukoplakia in epithelial cells of the stratified squamous epithelium of the oral mucosa increased proliferative activity of the cells with the expression in the cell nuclei Ki-67 protein and markers, that are directly (HPV16) or indirectly (P16INK4a) associated with human papillomavirus, is revealed. At hyperplasia with hyperkeratosis only protein P16INK4a is revealed, squamous intraepithelial neoplasia with hyperkeratosis and at squamous cell carcinoma p16INK4A and antigens of HPV of a person at high risk (HPV16) are detected.

Keywords: leukoplakia, oral mucosa, immunohistochemistry, human papillomavirus 16 type, Ki-67, P16INK4a.

1. INTRODUCTION

Leukoplakia, the most common precancerous lesion of the oral mucosa [1, 2], is a type of

mucous keratosis characterized by chronic course and lesions of the oral mucosa and red border of lips [3]. At pathologico-anatomic examination, in cases of a clinical diagnosis of «leukoplakia», in 80-85% of the situations, hyperkeratosis with hyperplasia of basal and spinous layers of the epithelium is detected, hyperkeratosis with different degree of dysplasia is detected in 5-15% of the cases, while squamous cell carcinoma is detected in 2-5% of the cases [4].

Development of leukoplakia is caused by various polyetiologic factors, such as mechanical, chemical and thermal injuries. Approximately 70-90% of the cases of oral leukoplakia lesions are associated with smoking. Also, there is a direct correlation between the frequency and duration of smoking and the development of leukoplakia [5, 6].

At present, development of leukoplakia is related with the presence of human papilloma virus (HPV) [7], a small DNA virus that reveals tropism by stratified squamous epithelium. About 120 types of HPV have been identified up to now. HPV with high oncogenic risk causes an estimated ratio of 40% of cancers of oral mucosa [8]. HPV virus replication is carried out only in the proliferating epithelial cells of the mucous membrane. As known, with the increase of the degree of dysplasia, the number of proliferating cells expressing Ki-67 protein in the epithelium of oral mucous membrane increases [9, 10]. Therefore, diagnosis of such cells by the immunohistochemical marker of proliferation Ki-67 can be used as an additional criterion of precancerous condition.

HPV activates the proliferative activity of epithelial cells by blocking the P16INK4a protein. The P16INK4a gene is located in the section of

chromosome 9p21. It contains three exons and encodes a nuclear phosphoprotein with a molecular weight of 16 kDa. The function of this gene protein is to inhibit the cell cycle by binding cyclin-dependent kinases 4 and 6, and to interact with cyclin D1 [11].

Considering all these, the **aim of the study** was to detect proteins Ki-67 and P16INK4a and the antigens of human papilloma virus of high oncogenic risk (HPV16) at hyperplasia, dysplasia and squamous cell carcinoma of the oral mucosa.

2. MATERIALS AND METHODS

The biopsy material of the oral mucosa of 82 patients - 42 women and 40 men affected with leukoplakia - was studied. Average age of patients: 62 years. For histological examination, unmodified epithelia, taken over from the areas of mucous membrane adjacent to leukoplakia, were used as control. Histological assessment of the test material was performed according to WHO classification (2005).

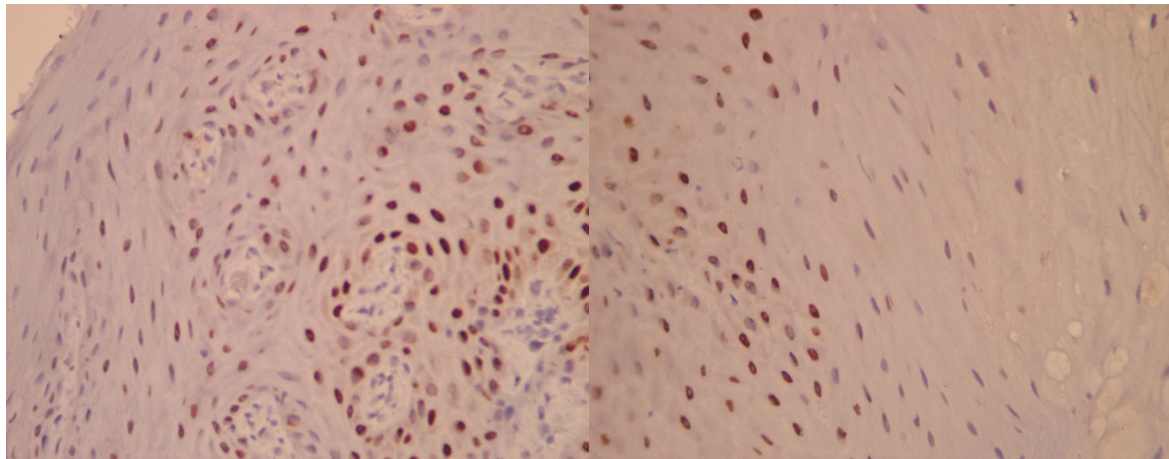
The biopsy material was fixed in 10% neutral formalin (pH 7.4). After their placement on the tissue processor, the samples were embedded into paraffin, at a melting temperature of 54°C. For histological and immunohistochemical (IHC) investigation, serial sections (5 micrometers thick) were prepared, then placed on glass slides coated with poly-L-lysine. Identification of tissue antigens was carried out by using rabbit's polyclonal antibodies to human papilloma virus of 16 type ("Thermoscientific") in a 1:400 dilution, and monoclonal antibodies P16INK4a (clon EPR1473, EPITOMICS) - 1:200 and Ki-67 (Thermoscientific) - 1:100. Detection of the immune complexes was carried out with the UltraVision Quanto Detecton System HRP

("Thermoscientific") detection system, with sections previously dyed with Mayer's hematoxylin. The reaction to the human papilloma virus of type 16 and P16INK4a was evaluated qualitatively by cellular zones: in the basal and spinosum cell layers, peripheral and central dividing tumor cell areas were evaluated at squamous cell carcinoma. The Ki-67 proliferation index (IPKi-67) is determined by the number of immunoreactive cell nuclei to the total number of nuclei. Evaluation of these indices is performed on appropriate tissue areas. Cell counts were carried out at a magnification of 400.

Statistical processing was carried out by Statistica10.0, using standard methods. Considering the abnormal distribution of the statistical indices, a comparison of two independent groups was carried out by the non-parametric method, with Mann-Whitney U-test. The differences at average level of statistical significance at $p < 0.05$ were considered as reliable. Using the Spearman coefficient r (Table 2), the correlation relationship between the proliferative activity of cells (for Ki-67 protein expression) in leukoplakia with symptoms of hyperplasia, dysplasia, squamous cell carcinoma, and the severity of the expression of human papilloma virus antigens (16-type) and P16INK4a, was studied.

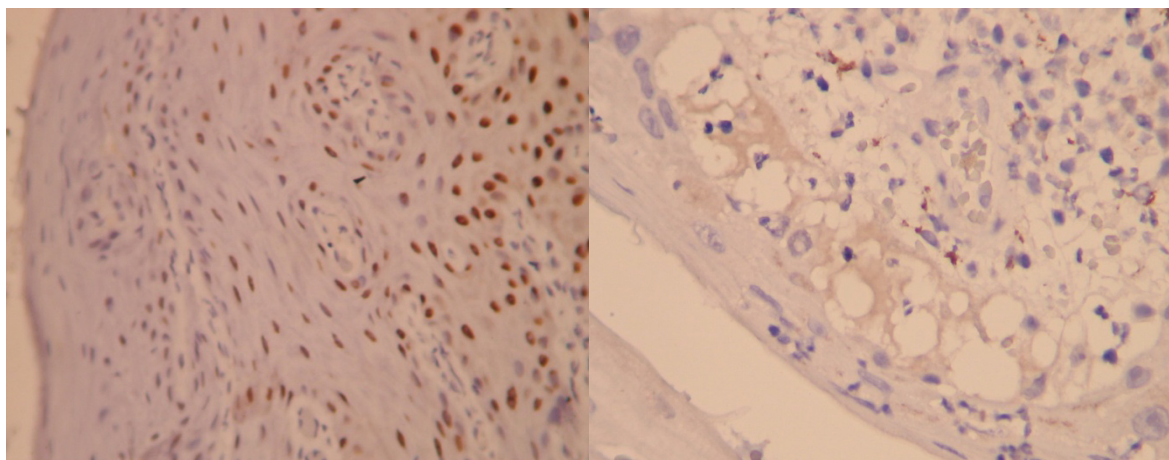
3. RESULTS AND DISCUSSION

During the study, 10 (12%) areas of the unmodified mucous membrane, 35 (43%) biopsy materials without atypia leukoplakia, 16 (20%) biopsy materials of leukoplakia with dysplasia of varying degrees, from SIN 1 to SIN 3, were studied; a squamous cell carcinoma was detected in 21 (25%) patients.



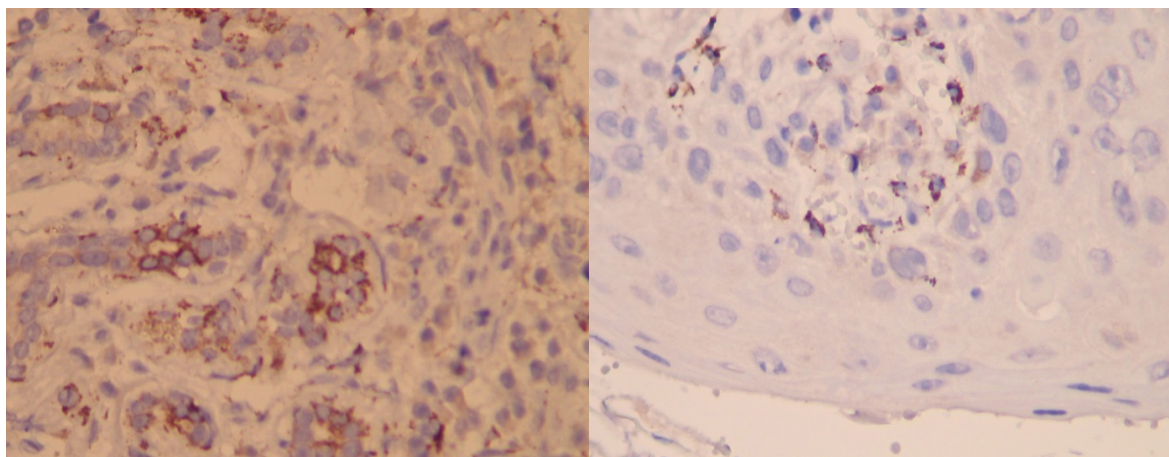
A

B



C

D



E

F

Fig. 1. IHC reaction in leukoplakia with hyperplasia of epithelium (a, b), SIN (c, d) and squamous cell carcinoma (e, f) oral mucosa with antibodies to P16INK4a (a, c, e) and HPV16 (b, d, f). DAB-Mayer's hematoxylin counterstain (a-c - x 120;d-f - 240)

IHC study of oral mucosa epithelium has revealed features of protein expression that characterize the proliferative processes and viral antigens during different morphological manifestations of leukoplakia showing human papilloma virus replication in the proliferating cells of the stratified squamous epithelium.

A IHC study of unmodified epithelium detected proliferating cells only in the basal layer. In 6 cases, in the nuclei of cells of all layers, P16INK4a protein was detected, and in 2 cases - protein HPV16. In the clinical material of leukoplakia with hyperplasia of mucous membrane and occurrences of hyperkeratosis, Ki-67 protein expression was detected in the nuclei of epithelial cells of the basal and spinous layers. With this type of pathological process, in 77% of cases, P16INK4a protein expression was observed in the nuclei of the stratified squamous epithelium of all investigated zones, and in 34% of cases - a positive reaction to viral antigens HPV16 was revealed. Fig. 1 (a, b) shows a positive IHC stain of the nuclei of epithelial cells at protein P16INK4a and

negative reaction to viral antigens HPV16. At leukoplakia with SIN symptoms, the number of proliferating cells was increased in the parabasal and spinous layers of the epithelium (Table 1). In the same zones, in 75% of cases, P16INK4a protein expression was detected (Fig. 1, c) and in 50% - small inclusions of HPV16 antigens in the cell nuclei and cytoplasm (Fig. 1, d.) were also detected. At squamous cell carcinoma, in the peripheral and central zones, the amount of proliferating cells was significantly increased compared to hyperplasia and SIN. In the same zones, in 86% of cases, a high nuclear expression protein P16INK4a was revealed, in 62% of cases - inclusion of HPV16 antigens in the cell nuclei and cytoplasm (Fig. 1 e, f.) was detected.

Statistical analysis revealed a high correlation between the proliferation of cells in the parabasal layer at leukoplakia with hyperplasia and expression P16INK4a protein, as well as leukoplakia with dysplasia in parabasal, spinous cell layers and the presence of HPV16 antigens (Table 2).

Table 1. Ratio of the expression of Ki-67, P16INK4a and HPV16 antigens in various morphological variants of oral leukoplakia

№ №	Performance	n	Ki-67 (basal layer)	Ki-67 (parabasal layer)	Ki-67 (spinous layer)	P16INK4a, %	HPV16, %
			M±SD, %				
1	Unmodified epithelium	10	92.1±3.6	0	0	60	20
2	Leukoplakia with hyperplasia	35	58.5±36.2*	38.4±24.1*	0.3±1.7*	77	34
3	Leukoplakia with SIN	16	45.9±33.1*	38.1±21.5*	4±14.7*	75	50
4	Squamous cell carcinoma	21	Peripheral		Central	Peripheral	Central
			60.5±25.3*		39.3±30.6*	86	62

*-- p<0.05.

Table 2. Spearman correlation coefficient between the percentage of proliferating cells and expression P16INK4a and HPV16 antigens in various morphological variants of oral leukoplakia

Performance	n	Ki-67 (basal layer)		Ki-67 (parabasal layer)		Ki-67 (spinous layer)	
		rs, p					
		P16INK4a	HPV16	P16INK4a	HPV16	P16INK4a	HPV16
Unmodified epithelium	10	-0.284 0.426	-0.522 0.1215	0 1.000	0 1.000	0 1.000	0 1.000
Leukoplakia with hyperplasia	35	-0.317 0.064	-0.474 0.004	0.397 0.018	0.228 0.187	0.166 0.339	-0.030 0.863
Leukoplakia with SIN	16	0.235 0.380	0.298 0.260	0.094 0.728	0.515 0.041	0.266 0.318	0.651 0.006
Squamous cell carcinoma	21	Peripheral				Central	
		P16INK4a	HPV16		P16INK4a	HPV16	
		0.022 0.923	0.390 0.080		-0.157 0.495	-0.211 0.358	

Studies have shown that, at different morphological variants of leukoplakia from hyperplasia with hyperkeratosis to squamous cell carcinomas in the epithelial cells of the stratified squamous epithelium, high-risk oncogenic human papilloma virus antigens and HPV16 proteins associated with HPV (P16INK4a) were found. The P16INK4a protein hinders tumor formation by blocking cyclin-dependent kinase (cdk4), while cdk6-caused phosphorylation of pRb, leading to the inhibition of E2F-dependent transcription and realization of the cellular cycle from the points of comparison G1 to S [12]. Inhibition of gene expression by P16INK4a at the expense of hypermethylation or mutations is often observed in most cancer cell lines and primary human tumors. Thus, increased expression of the gene is an indirect P16INK4a HPV marker that displays violation mechanisms controlling cell proliferation, being characterized by persistence of infection with a high risk of developing neoplasia [13, 14].

Considering that, currently, more than 120 types of HPV have been identified, detection of their antigens by the IHC-technique is practically impossible. This may explain the low detection

rate of HPV antigens of 16 type at hyperplasia with hyperkeratosis, against the background of a high expression P16INK4a, which is most likely caused by papilloma viruses of other types.

In these studies, a significant correlation between SIN and HPV-16 type was revealed. However, data about the presence of HPV-infection in epithelial cells of oral mucosa at precancerous and cancerous lesions is quite contradictory, ranging from 0 to 100% [15, 16]. The reason of these discrepancies most likely lies in the methodical features of sample selection, HPV research methodologies, etc. [17].

4. CONCLUSIONS

For various morphological variants of leukoplakia in the epithelial cells of the stratified squamous epithelium of the oral mucosa, increased proliferative activity of the cells with the expression in the cell nuclei Ki-67 protein and markers, that are directly (HPV16) or indirectly (P16INK4a) associated with the human papilloma virus, is revealed. In cases of hyperplasia with hyperkeratosis, only protein P16INK4a is

revealed, and squamous intraepithelial neoplasia with hyperkeratosis and at squamous cell carcinoma p16INK4A and antigens of HPV of a person at high risk (HPV16) are detected.

Detection of human papilloma virus infection at leukoplakia can not only identify its genesis, but can also become a morphological basis for effective prevention and treatment of common diseases of the oral mucosa.

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