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EXPRESSION AND LOCALISATION OF CD44 ANTIGEN AS A PROGNOSTIC FACTOR OF ORAL LEUKOPLAKIA

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It is essential to identify markers that could indicate the presence of early molecular changes in premalignant tissues like oral leukoplakia (OL). CD44 adhesion molecule is not only a stem cell marker, but also determines cell proliferation and migration in malignant processes. The aim of our study was to assess the amount and pattern of CD44 antigen expression by epithelial and mononuclear cells in the lamina propria under OL and their role in premalignant lesions. The current study included 102 cases of OL and ten biopsies from healthy oral mucosa. Immunohistochemical CD44 antigen expression was determined in 34 cases by a standard EnVision imaging system in three points of OL: both edges and centre. Statistical analysis was done using GraphPad Prism software version 8.4.0. In OL, statistically significant overexpression of membranous CD44 was demonstrated compared to healthy mucosa ($p < 0.0001$). The intra-cytoplasmatic CD44 expression of epithelium together with characteristic nuclear changes may be used as a predictive factor for potential malignant transformation of non-homogenous leukoplakia. CD44 expression in mononuclear cells under the basal membrane in OL ($p < 0.05$) possibly influences the process of premalignant lesion transformation into intraepithelial cancer. Further study of CD44 antigen expression in intra-cytoplasmatic structures is required for better explanation of the role of this glycoprotein.

Key words: *cell-surface glycoprotein, oral precancers, mononuclear cells of lamina propria.*

INTRODUCTION

Globally, 4% of all cancer-related deaths are attributed to mouth, pharynx and larynx, and they are the 8th most common cause of death from cancer. In the Baltic countries, oral cavity cancer composes 1.1 to 2% of all malignancies and 5-year prevalence varied from 19.74% in Estonia to 37.98% in Latvia (Anonymous, 2018). In the Northern Europe countries, including Latvia, in recent years young people have increased usage of smokeless tobacco, which along with alcohol consumption, smoking, and chronic inflammatory processes of oral mucosa are possible causes of potentially premalignant disorders and oral malignancies. The most common precursors of oral squamous cell cancer are oral leukoplakia, erythroplakia, submucosal fibrosis and erosive and atrophic forms of oral lichen ruber planus (Renaud-Vilmer and Cavelier-Balloy, 2017; Sundberg *et al*., 2019).

The role of oral leukoplakia (OL) in the development of oral cancer has been discussed in literature for many decades (Roye *et al*., 1996; Wang *et al*., 2009; Garcia *et al*., 2019). There have been attempts to identify other risk factors for malignant transformation of OL into cancer: leukoplakias mainly of tongue or of the floor of mouth, its size larger than 2 cm, and the non-homogeneous type (Assimakopoulos *et al*., 2002; Speight *et al*., 2018). However, most scientists and dentists agree that the key factor for malignant transformation processes of squamous epithelium in different organs is severe dysplasia (Herold-Mende, 1996;

Alsalem, 2019; Naga *et al*., 2019). The transformation rate for oral leukoplakia into oral cancer in different parts of the world is from 1–12% (Narayan and Shilpashree, 2016; Warnakulasuriya, 2019). Important studies have shown that OL may transform into squamous cell cancer in approximately 57 months. From the point of view of normal histology, progenitor epithelial cells reach the surface of buccal mucosa in 25 days, gingival — in 50 days, whereas in junction areas — rapidly in 5–6 days (Nanci, 2017, p. 80). Thus, by biopsy medical staff capture only one moment in the complicated course of hyperplasia and dysplasia of lining mucosa. Animal models are not always acceptable for the understanding of this long-term and complicated process that leads to invasive carcinoma (Wielenga *et al*., 1993; Herold- Mende *et al*., 1996)

However, for clinicians in dentistry it is still very important to provide clear information about possible morphologic and immunohistochemical predictive factors in oral leukoplakias and cancer (Godge and Poonja, 2011). Malignancies involve important processes like proliferation, migration, and loss of the adhesion of cells, and one of the molecules reflecting these processes is CD44 antigen. During the last years, CD44 has received names that describe its biochemical features: antiheparan sulfate proteoglycan, antihyaluronate receptor antibody, hyaluronic acid receptor, stem cell marker and anti-phagocytic glycoprotein I. Most of the studies about the role of CD44 are associated with malignancies. The CD44 family has more than 30 subtypes. Some of its variants have been analysed in animal models (Pals *et al*.,1993; Wielenga *et al*., 1993), in biopsy and surgical material of malignancies in lymph nodes, skin, esophagus, brain (Roye *et al*., 1996; Pettersen *et al*., 2011; Jakovlevs *et al*., 2019) and particularly in head and neck tumours (Boxberg *et al*., 2018; Tamatani *et al*., 2018). Correlation between CD44 expression and prognosis, grade, spread and T stage of oral squamous cell cancer has been demonstrated (Kaza *et al*., 2018; Chen *et al*., 2019). However, scientists have mainly described a decreased amount of CD44 adhesion molecules in oral cancer and only some studies have demonstrated an increased amount of this antigen in malignancies of the oral cavity (Lindquist *et al*., 2012).

The role of CD44 antigen in oral leukoplakia is still controversial. A few researchers have found an increased amount of CD44 antigen in OL (Godge and Poonja, 2011), whereas most have diagnosed a decreased amount of CD44 by calculating number of CD44-labelled cells manually, automatically using traditional immunohistochemical methods with immunoperoxidase, using Western blot analysis and even flow cytometry (Miletti-Gonzalez *et al*., 2012; Chen *et al*., 2019).

In the last years, CD44 antigen has been named also as a stem cells marker of different organs (Pettersen *et al*., 2011; Tamatani *et al*., 2018; Jakovlevs *et al*., 2019). However, it is important to emphasise that there are three types of stem cells: embryonic, adult and cancer ones. In oral leukoplakias and cancers there are possibly adult and cancer stem cell variants, now called also tumour initiating cells (Naga *et al*., 2019).

Participation of CD44 glycoprotein/anti-phagocytic glycoprotein I of lymphocytes and macrophages in various inflammatory processes and autoimmune diseases in humans and laboratory animals has also been described (Gore *et al*., 2008; Johnson and Ruffell, 2009). CD44 molecules play an active role in the immune reactions of nearby affected tissues of pulp, skin, brain and other organs (Pisterna and Siragusa, 2007; Pettersen *et al*., 2011; Jakovlevs *et al*., 2019). Research groups have established a role of CD44 in leucocyte recruitment to inflammatory sites and oral malignancies (Groma *et al*., 2012). These studies also revealed a role of CD44 in limiting the inflammatory response and resolving this process (Gore *et al*., 2008; Johnson and Ruffell, 2009).

There have been attempts of using anti-CD44 in experimental and practical medicine for the treatment of wounds, inflammation, and tumours (Negi *et al*., 2012; Reid *et al*., 2019).

The aim of our study was to assess the amount and pattern of CD44 antigen expression by epithelial and mononuclear cells atoral leukoplakias and their role in potentially premalignant lesions.

MATERIALS AND METHODS

Study group. The biopsies and surgical material of 102 patients with oral leukoplakia treated in the Department of Oral Pathology, Rīga Stradiņš University Institute of Stomatology and Centre of Maxillo-facial Surgery, Pauls Stradiņš Clinical University Hospital was used in the current study. The study included tissue samples examined by two independent pathologists after hematoxylin-eosin staining. Ten biopsies from healthy oral mucosa were considered as a control group. Gross and histopathological records of the Pathology Institute, Pauls Stradiņš Clinical University Hospital, were used to analyse the location and size of oral leukoplakia, patients' age, gender and previous anamnesis of oral diseases. Our study was designed according to the Helsinki Declaration. The study protocol was approved by the Committee of Ethics of the Rīga Stradiņš University.

Morphological examination. Surgical material and biopsies of oral mucosa were fixed in 10% neutral (pH = 7) buffered formalin solution, processed in a "Sakura Tissue-Tek VIP 5"^{TM} vacuum infiltration tissue processor and embedded in paraplast (Diapath, Bergamo, Italy). All biopsies from healthy and affected oral mucosa were stained with haematoxylin-eosin. Paraffin blocks were cut in 4 μm-thick sections; samples were placed on adhesive positively charged slides. Then dewaxing and rehydration through alcohols to water was done.

Immunohistochemical examination. Immunohistochemical CD44 antigen expression was determined by a standard polymer-based visualisation system (EnVision

method by Dako Denmark. clone DF1485, dilution 1 : 50. For the detection of CD44 glycoprotein, 42 samples from 34 oral leukoplakias were chosen (two pieces of mucosa were analysed from each surgical material), as well as ten cases with healthy mucosa. For immunohistochemistry all slides were incubated with 3% H_20_2 for ten minutes to inhibit endogenous peroxidase activity. The micro-wave-based antigen retrieval was performed in a freshly prepared 0.01 mol/l sodium citrate buffer (pH 6.0) solution at 750 W for three cycles, for ten minutes each. Specimens were immunostained using the primary antibody pan-CD44. Slides were counterstained with Mayer's hematoxylin, dehydrated in alcohol, cleared in xylene and cover-slipped. CD44 antigen expression in epithelial cells and mononuclear cells of lamina propria was considered positive if more than 10% cells expressed a positive reaction. CD44 expression was diagnosed in lymphocytes, monocytes and macrophages. The number of mononuclear cells in lamina propria mucosae area under leukoplakia and epithelial cell layers with CD44 antigen expression were counted in three fields of vision using original magnification of 400× at three different points of leukoplakia (both edges-locus 1 and 3 and central area-locus 2). Expression of CD44 was evaluated semi-quantitatively as weak, mild, and strong — 1st, 2nd, and 3rd level of immunoexpression. Oral leukoplakia was classified into homogenous and non-homogenous according to the WHO classification, taking into consideration its level of dysplasia — mild, moderate and severe. All slides were examined and photographed with a Leica Microscope (Leitz, Wetzlar, Germany).

Statistical analysis. Calculations, statistical analyses, and graph production were performed using the GraphPad Prism software version 8.4.0 for Mac (GraphPad Software, San Diego, CA, USA). The normality of numerical data was checked using D'Agostino and Pearson and Shapiro-Wilk tests, and if the data were not distributed normally, the non-parametric Mann–Whitney test was used to compare two groups. To compare three groups, the non-parametric Kruskal–Wallis (KW) test followed by the two-stage step-up method of Benjamini, Krieger, and Yekutieli as the post-hoc procedure was applied. Mean levels of the parameters were expressed as medians with dispersion, characterised by the interquartile region (IQR). Depending on the data distribution, the correlation between variables was determined by the parametric Pearson's test (CD44 expression in leukoplakia between three loci of epithelium) or by the non-parametric Spearman's test (CD44 expression in leukoplakia and in normal mucosa; mononuclear cells in leukoplakia and normal mucosa). The correlation between the number of CD44 labelled epithelial layers and monocytes in leukoplakia was determined by the Spearman's test. A p-value less than 0.05 ($p < 0.05$) was considered as a statistically significant difference.

RESULTS

Clinical and morphological characteristics. We analysed biopsy and surgical material from 102 cases of oral leukoplakia. The mean age of patients was 52.9 ± 14 (range of 25–72) years. The ratio between male and female patients was 11 : 1. Oral leukoplakias were localised in 54.6% of cases in buccal mucosa, 18.1% — on tongue and equally 9.1% on gingival mucosa, floor of the mouth and palate. The size of the oral leukoplakias varied from 4 mm to 30 mm, with average size 14 mm ± 3.7 mm. In females the size of oral leukoplakias was around 5 mm, but in males the average largest diameter of OL was 27 mm. Non-homogenous leukoplakias were diagnosed in 10.5%, whereas others were homogenous (89.5%). Oral epithelium of non-homogenous leukoplakias was characterised by mild and moderate dysplasia with characteristic changes of nuclei and altered nucleo-cytoplasmatic ratio.

Immunohistochemical CD44 antigen characteristics. In healthy mucosa, CD44 expression was positive in the membranes with an average of five layers of epithelium, mainly in the basal and in the intermediate layers but absent in the surface cells. The mean number of CD44 labelled cells in lamina propria mucosae of the control group was five. In the homogenous oral leukoplakia, membranous expression of glycoprotein was present in an average 17 layers of epithelium and had the 1st and 2nd intensity level (Fig. 1) and this difference was statistically significant ($p < 0.0001$) (Fig. 2A). Three points in healthy mucosa and leukoplakia were compared. In normal mucosa, CD44 expression in epithelium did not statistically significantly differ between three points of the samples (Fig. 2B). There was a statistically significant difference between locus 1 and locus 2 ($p = 0.0224$) and between locus 2 and locus 3 ($p = 0.0019$) of oral leukoplakia. In the second locus-centre of OL, a

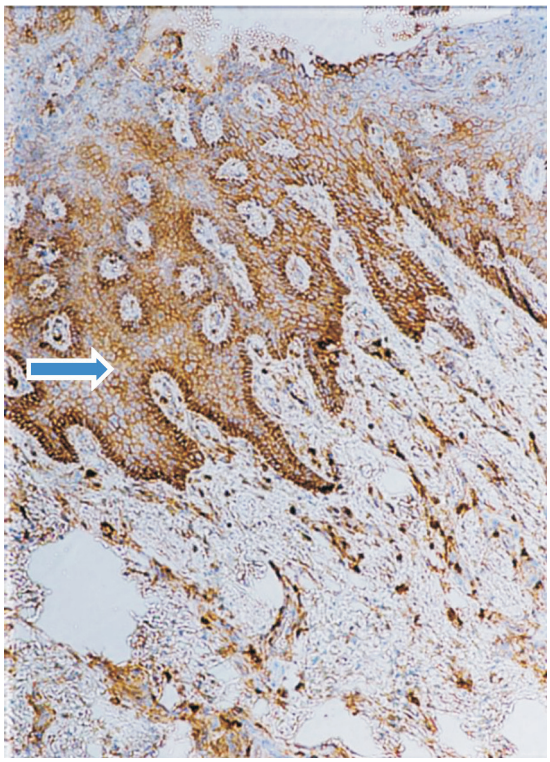

Fig. 1. Overexpression of CD44 antigen in multiple epithelial layers of oral leukoplakia (arrow), immunoperoxidase, anti-CD44, 100×.

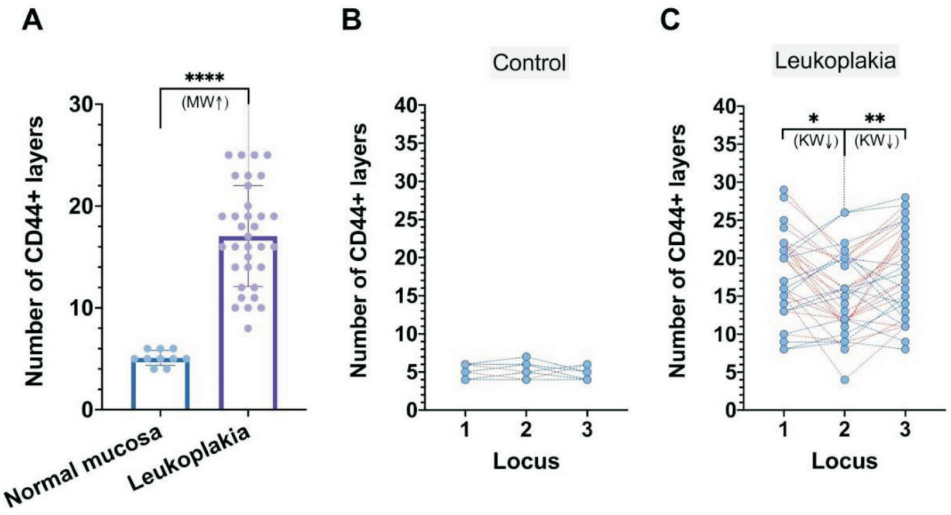

Fig. 2. A, Correlation of CD44 expression level in normal mucosa (control) and in leukoplakia. ****$p < 0.0001$; MW, Mann–Whitney test. B, CD44 expression in epithelium of normal mucosa in three different points; C, CD44 expression in epithelium of leukoplakia in three different loci; *$p < 0.05$; **$p < 0.01$; KW, Kruskal–Wallis test with Benjamini–Krieger–Yekutieli post-hoc procedure.

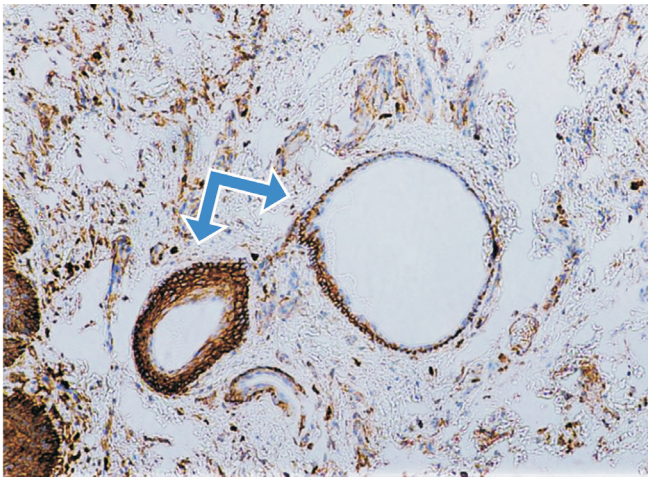

Fig. 3. Different expression of CD44 antigen in the ducts and acini of submucosal small salivary glands (arrows), Immunoperoxidase, anti-CD44, 100×.

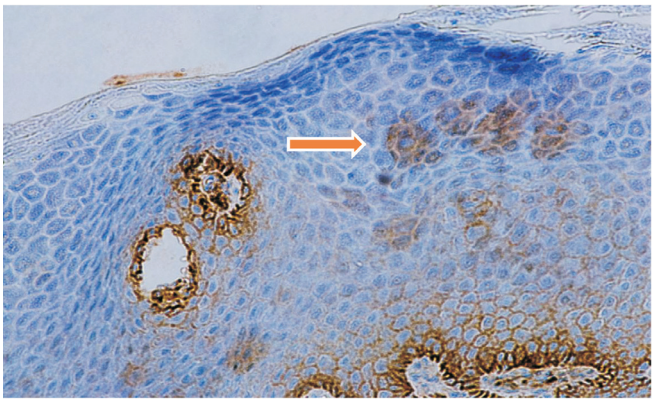

Fig. 4. Membranous and intra-cytoplasmatic presence (arrow) of CD44 glycoprotein in the epithelium of oral leukoplakia, immunoperoxidase, anti-CD44, 400×.

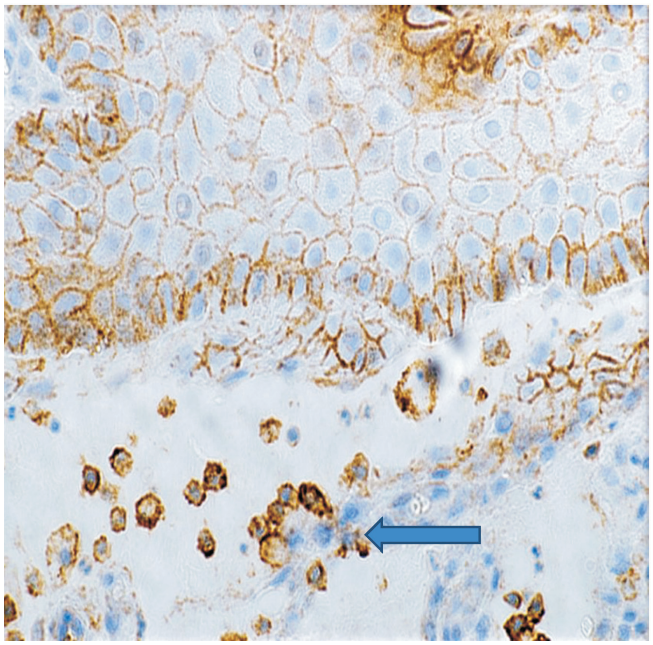

Fig. 5. Few mononuclear cells with CD44 expression in lamina propria of oral mucosa (arrow), immunoperoxidase, anti-CD44, 400×.

smaller number of CD44 positive epithelial layers was observed (Fig. 2 C).

In larger samples of surgically removed oral leukoplakias, CD44 was expressed positively in ducts and acini of small salivary glands. The amount of CD44 antigen was lower in the dilated ducts with flattened atrophic epithelium (Fig. 3). In 47% of non-homogenous oral leukoplakia where hyperplasia and dysplasia were present, there was not only membranous expression of CD44, but this glycoprotein also occurred in cytoplasm of the affected epithelium (Fig. 4). CD44 expression was detected also in the mononuclear cells under the basal membrane. In healthy oral mucosa, the number of the labelled macrophages and leucocytes was on average 5 in one field of vision (Fig. 5), with no significant correlation between three points of the biopsy (Fig. 6B). Under the leukoplakia, the number of CD44 labelled stromal cells was on average 18 (Fig. 7) and the difference was statistically significant ($p < 0.0001$) in comparison with the control samples ($p < 0.0001$) (Fig. 6A). Moderate correlation ($p = 0.0045$) between CD44 positive epithelial layers and mononuclear cells of lamina propria mucosae with its expression in the second locus (central part of leukoplakia) was observed (Fig. 7C). We detected activation of stromal

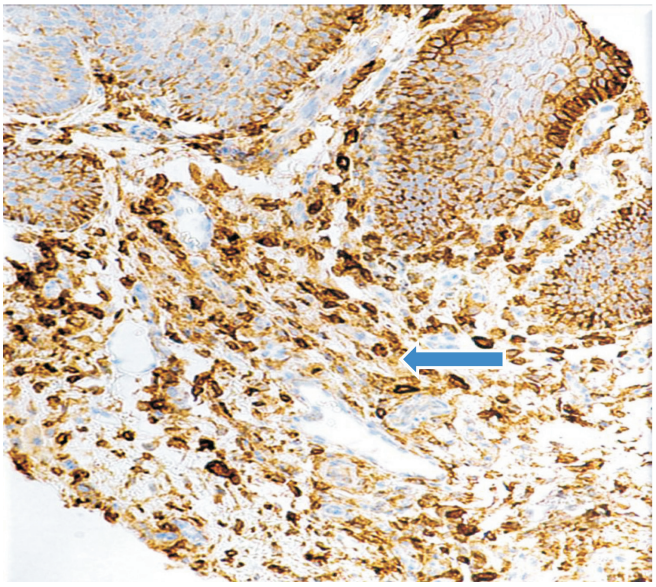

Fig. 6. A, number of CD44 positive mononuclear cells in healthy mucosa and leukoplakia samples. $p < 0.0001$; MW, Mann–Whitney test; B, Mononuclear cells of normal mucosa in three different points; C, Mononuclear cells in lamina propria under leukoplakia in three different points.

cells in lamina propria mucosae especially under the central part of oral leukoplakia and observed that CD44 antigen accumulates simultaneously in the epithelium and lamina propria cells. This correlation was confirmed by the non-parametric Spearman's test (Fig. 8).

DISCUSSION

In the Baltic countries, oral and pharyngeal cancer is still diagnosed at the advanced stages of the disease. Based on the EUCAN National register data, the highest incidence of the oral and pharyngeal cancer in the Northern European countries is in Denmark — 910 (13.0), which is much higher than in the Baltic states: Lithuania 387 (9.9); Estonia 143 (8.5) and Latvia — 215 (7.5). In terms of mortality, the highest rates for 2012 were reported in Lithuania 286 (7.5) followed by Estonia 110 (6.5) and Latvia 149 (5.4) (Diz *et al*., 2017). This means that it is essential to identify markers

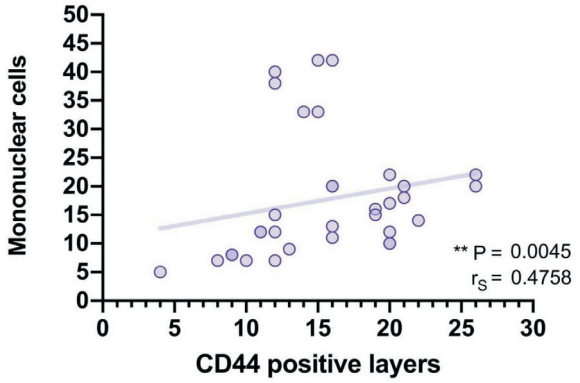

Fig. 8. Correlation between the number of CD44 labelled epithelium layers and mononuclear cells in lamina propria under the central area of leukoplakia (locus 2) performed by non-parametric Spearman's test.

that could indicate the presence of early molecular changes in such potentially premalignant tissues as oral leukoplakia.

In the European countries and USA, OL transforms into OSCC less commonly than in Asia (Narayan *et al*., 2016; Speight *et al*., 2018; Alsalem, 2019). It is important to emphasise that potentially premalignant lesions and malignancies of the oral cavity belong to the self-examinable neoplasia, and patients themselves must be involved in the detection of visual or palpable changes of their oral mucosa. The most important role in the diagnostics of OL is played by a physician who must differentiate leukoplakias from other white epithelial and non-epithelial lesions of the oral cavity. Therefore, it is possible to exclude or accept potentially premalignant oral lesions only by analysis of diagnostic biopsies.

The patients of our study group were typically at the age between 50 and 70 years, and they were almost all male. Therefore, we conclude that men's lifestyle, harmful habits, different environmental factors, and oral hygiene differ a lot from those of females. The median age of the patients of our study corresponded to the data from other studies (Renaud-Vilmer and Cavelier, 2017; Naga *et al*., 2019). The size of leukoplakia may be very different; even multiple oral leukoplakias are described (Tamatani *et al*., 2018). In our opinion, it is important to underline not only the diame-

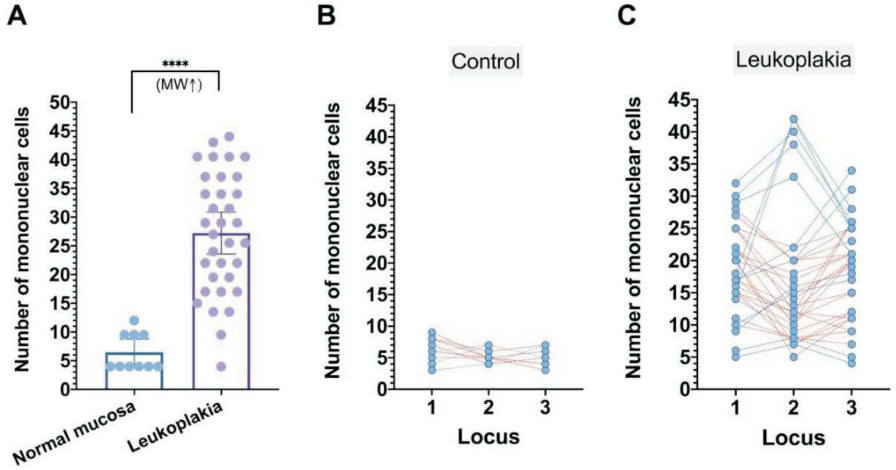

Fig. 7. A large number of laminae propria mucosae mononuclear cells with intensive expression of CD44 antigen in them under leukoplakia (arrow), immunoperoxidase, anti-CD44, 200×.

ter of a white lesion but also its thickness. In thicker OL, there is less maturation and differentiation of epithelium, worse microcirculation, changed cell cycle and injury of organelles, and therefore it is more likely that dysplasia will develop and progress. Particularly larger and thicker OL must be removed totally. In our group of patients, incomplete removal of OL was seen in 7% of cases. Conclusions of a pathologist in multiple studies showed recurrence of leukoplakia in 20% to even 73% of smokeless tobacco users (Godge and Poonja' 2011; Sundberg *et al*., 2019). CD44-*pan* antigen is one of the adhesion molecules realising cell to cell interactions also in oral leukoplakia, but it participates also in processes like inflammation, neoplasia, autoimmune diseases and others (Puré and Oaff, 2001). Oral cavity researchers had mainly concentrated on the role of CD44 as a predictive and prognostic factor of oral squamous cell cancer. Some research groups have found that overexpression of CD44 correlates with wider dissemination, greater T stage and higher grade (Otriz *et al*., 2011; Negi *et al*., 2012), but others have underlined that a decreased amount of CD44 is not associated with advanced stages of oral cancer (Boxberg *et al*., 2018). Most authors describe membranous location of the CD44 adhesion molecule, and only a few have diagnosed its expression in cytoplasm (Groma *et al*., 2012; Kaza *et al*., 2018). In oral leukoplakia, the results are even more controversial as different microscope magnifications have been used in pathohistological examinations, various fields of vision and even contrasting methods of counting. Our study group chose to count the number of oral epithelia labelled with CD44 in each leukoplakia, as this represents adhesion molecules in leukoplakias of different thickness. Our statistical analyses showed a statistically significant difference ($p < 0.0001$) between the number of CD44-labelled layers in normal and affected mucosa. Therefore, we recommend this method of counting for better evaluation of CD44 expression in studies of potentially premalignant lesion and early carcinomas of the oral cavity. We have showed CD44 intra-cytoplasmatic immunoexpression in 47% of non-homogenous OL. This phenomenon was shown in several studies of oral squamous cell cancer (Khajuria and Metgud, 2015). Only a few authors have described interaction of CD44 with the cytoskeleton (Harada and Takahashi, 2007; Miletti-Gonzalez, *et al*., 2012). In our opinion, this fact possibly demonstrates the expression of glycoprotein CD44 in the injured mitochondrion, endoplasmatic reticulum or Golgi complex of hyperplastic and dysplastic epithelium of oral leukoplakia, which needs to be verified by electronmicroscopic examination of such leukoplakias. As CD44 is also a marker of stem cells, membranous expression of glycoprotein occurs in the adult stem and amplifying cells of oral mucosa whereas cytoplasmatic labelling may be present in single cells of oral leukoplakias. The pattern of CD44 expression in the cytoplasm of oral epithelium may be used as a predictive factor for potential transformation of non-homogenous leukoplakia into an early stage of cancer. The difference between the adult stem cells and the so-called cancer stem cells is fragile and unstable, and can be demonstrated only by immunohistochemistry, therefore, studies of oral leukoplakias should include their genetic examinations (Ambele *et al*., 2020). The CD44 antigen can bind growth factors, hyaluronic acid, collagen, fibronectin, and metalloproteinases, and participation of CD44 in lymphocyte and macrophage activation, leucocyte rolling and aggregation has been described (Gore *et al*., 2008; Alves *et al*., 2009). Therefore, it is of scientific interest that our results showed a statistically significant difference ($p < 0.0001$) between the mononuclear cells level in the lamina propria of normal mucosa and leukoplakia samples. There was an increased number of mononuclear cells (macrophages, monocytes, and lymphocytes) under the basal membrane in the area of OL and normal oral mucosa. Our statistical analyses showed moderate correlation between the amount of CD44-positive mononuclear cells in the lamina propria mucosae and number of CD44-labelled oral epithelium layers in the central part of leukoplakia where more likely the most active interactions between adhesion molecules are found ($p = 0.0045$; $r = 0.4758$). Simultaneous accumulation of CD44 antigen in the oral epithelium and stromal cells under leukoplakia ensures its participation in the local immune reactions, in long-term epithelial- mesenchymal interactions as a hyaluronic receptor protein and antiphagocytic glycoprotein.

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CD44 ANTIGĒNA EKSPRESIJA UN TĀ LOKALIZĀCIJA KĀ ORĀLĀS LEIKOPLAKIJAS PROGNOSTISKAIS FAKTORS

Turpinās šūnu marķieru izpēte, kuri norādītu uz agrīnām molekulāram pārmaiņām potenciālos vēždraudes audos, piemēram, mutes leikoplakijā (ML). Mūsu pētījuma mērķis bija noteikt CD44 ekspresijas daudzumu un tā veidu ML epitēlijā, izvērtējot tieši epitēlija slāņu skaitu un gļotādas saistaudu mononukleārās šūnās un novērtēt glikoproteīna lomu pirmsvēža bojājumos. Pētījumā iekļāvām 102 leikoplakiju biopsijas un operāciju materiālus; kontroles grupā bija 10 normālas gļotādas biopsijas. Imūnhistoķīmisku CD44 ekspresiju noteica ar standarta vizualizācijas sistēmu EnVision 42 paraugos no 34 ML. Statistiskā analīze veikta, izmantojot GraphPad Prism programatūru 8.4.0 versiju. Leikoplakiju gadījumos tika pierādīta statistiski ticama CD44 antigēna palielināta ekspresija epitēlijā, salīdzinot ar neizmainītu gļotādu ($p < 0.0001$). CD44 ekspresija mutes epitēlija citoplazmā kopā ar raksturīgām šūnu kodolu izmaiņām var būt kā prognozējošs faktors tieši nehomogēnas leikoplakijas potenciālai malignizācijai. Sinhrona CD44 antigēna palielināšanās mononukleārās šūnās zem bazālās membrānas leikoplakijas apvidū ($p < 0.05$), iespējams regulē vēždraudes audu tranformāciju intraepiteliālā mutes gļotādas vēzī, bet ir nepieciešama tālāka CD44 ekspresijas izpēte epitēlija intracitoplazmatiskajās struktūrās, lai labāk izskaidrotu šī glikoproteīna lomu.