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The Significance Of Immunohistochemical Expression of CD44 And Ki67 In Patients With Oral Leukoplakia.

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ABSTRACT

Oral leukoplakia (OL) is a potentially malignant lesion of the oral mucosa with increased risk of malignant transformation compared to healthy mucosa. Today, many clinical, histopathological, and molecular factors are used as potential predictors of malignant transformation. The aim of the research is to immunohistochemically determine the presence of CD44 and Ki67 in OL and to compare the obtained data with the presence of CD44 and Ki67 in the oral squamous cell carcinoma (OSCC) and healthy oral mucosa (HOM). Thirty OL, 30 OSCC and 20 HOM were used for immunohistochemical analysis. Significant difference between groups in relation to the presence of CD44 and Ki67 in the epithelium were detected. Significant difference between groups in relation to the presence of CD44 in II were detected. Significant difference of Ki67 presence in II has been detected between OSCC and OL, OSCC and HOM, but not between OL and HOM. Positive correlations with CD44 both in the epithelium and in the II of OL were detected. Positive correlation between CD44 in inflammation with CD44 and Ki67 in the epithelium of OL were detected. Increased immunohistochemical presence of CD44 and Ki67 in OL may be a useful predictor of early changes in malignant transformation.

Keywords: oral leukoplakia, oral squamous cell cancer, molecular markers, Ki67, CD44.

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INTRODUCTION

Oral leukoplakia (OL) displays similar proliferative characteristics to the oral squamous cell carcinoma (OSCC). According to the World Health Organization, OL is a precancerous lesion of the oral mucosa clinically described as a white lesion, which cannot erased or classified into other oral disease [1]. Today, OL considered as one of the most significant precancerous lesions regarding OSCC development. The malignant transformation rate of OL ranges between 0.7% and 2.9% [2]. The presence of dysplasia indicates malignant potential of OL, but some OLs will never malignantly transform, while some without dysplasia will evolve into OSCC [3]. So far, numerous molecular markers studied in order to determine which OL will become OSCC. However, there is still no widely accepted specific biomarker, which will predict the risk for OL malignant transformation [4]. Ki67 is one of the most important proliferative marker, which indicates the proportion of malignant cells during the cell growth and reproduction phase. It's higher values correlate with an earlier tumour recurrence and worse overall survival of the patients [5]. The Ki67 recognized as useful biomarker of different phases of the cell growth, being low during the G1 and early S-phase, and progressive increase during the mitosis [6]. Furthermore, numerous studies to date have introduced Ki67 as relevant in prognosis of lung and breast cancer [7,8].

Previous studies suggested difference of Ki67 expression in oral mucosa malignant transformation between normal epithelium, precancerous lesions and OSCC. Results of the studies have shown that Ki67 expression was the highest in oral cancer, lower in mild and moderate dysplasia and OL, and lowest in normal oral mucosa [9,10]. Several studies have shown that p53 and Ki67 are indicators of unfavourable prognosis in OL [11-13]. Contrary to that, there are studies, which results have shown that Ki67 is weak marker for the malignant transformation in the oral tissues [14-16].

CD44 is part of family transmembrane glycoproteins that expressed in different cells and is involved in numerous cellular processes. In addition, it participates in pathological processes of tumour growth, tumour proliferation and tumour cell dispersal [17]. Immunohistochemically proven increased CD44 expression was also observed in other tumours, like ovarian and colorectal, which correlated with less differentiated cancers as well as with less favourable prognosis [18,19]. There are cancer stem cells (CSCs) in tumour tissue that are undifferentiated and exhibiting high proliferative capacity due to overexpression of stem markers and consequently affecting tumour metastasis, drug transport, and the mechanism of DNA repair. It is assumed that CSCs are responsible for the onset, progression, recurrence, tumour invasion, and tumour resistance to treatment [20]. The CD44 glycoprotein called a marker of subgroup of CSCs, which possess the ability of self-renewal [21]. Overexpression of transmembrane glycoprotein CD44 in undifferentiated cancer stem cells predicts progression, recurrence, neoadjuvant therapy resistance and treatment success in patients with OSCC [22]. In the early 1990s variants of CD44 was identified, specifically v6 isoform which regulate tumour progression, invasion and metastasis [23]. To date only few studies have investigated expression of CD44 in oral precancerous lesions [24].

Therefore, the aim of this study is to immunohistochemically determine expression of Ki67 and CD44 in OLs and OSCCs.

MATERIAL AND METHODS

We performed retrospective cross-sectional analysis on archive tissue samples from the patients with a confirmed diagnosis of OL and OSCC collected at Clinical institute of pathology and cytology, Clinical hospital Sisters of mercy in Zagreb between 2013 and 2018. The study includes 30 histologically confirmed OLs, 30 histologically confirmed OSCCs. The control group consisted of 20 healthy oral mucosa (HOM) in which no histopathological changes described by histological analysis. Study approved by Ethics Committee of Clinical hospital Sisters of mercy and School of Dental medicine, University of Zagreb. All procedures performed in study were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments.

Immunohistochemistry

The archival material fixed in 10% buffered formalin and embedded in paraffin blocks. For immunohistochemical analysis, each section was thermally deparaffinised and incubated with the appropriate primary antibody, a mouse monoclonal antibody on Ki67 (MOA-HU Ki67, clone MIB-1, 1: 150, DAKO) and mouse monoclonal antibody to CD44 (MO A-HU CD44, clone DF 1485, 1:50, DAKO).

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Immunohistochemical analysis for antibodies was determined by the LSAB method as a visualization system on a Dako TechMate TM Horizon (Dako, Copenhagen, Denmark) an automated immunohistochemical staining machine. The time and mode of thermal detection of the epitope, the dilution and the incubation time of each antibody performed according to the manufacturer's protocols. Secondary antibody reaction visualization was done with En Vision HRP rabbit / mouse ENV (K5007, DAKO) and chromogenic diaminobenzidine (K3468, DAKO), and counterstained with Mayer haematoxylin.

The preparations were analysed by light microscope at low (40x) and high (400x) magnification.

The immunohistochemical reaction to CD44 was determined semi-quantitatively according to the method described by Ghazi et al. [25]:

0 - no positive membrane response or less than 5% of cells,
1+ poorly positive membrane reaction; 5-25% of cells,
2+ moderately positive membrane reaction; 25-50% of cells,
3+ medium positive membrane reaction; 50 -75% of cells,
4+ highly positive membrane reaction; more than 75% of cells.

The immunohistochemical positivity of nuclei on Ki67 will be determined semi-quantitatively according to the method described by Ghazi [25]:

0 - no positive membrane response or less than 5% of cells,
1+ poorly positive membrane reaction; 5-25% of cells,
2+ moderately positive membrane reaction; 25-50% of cells,
3+ medium positive membrane reaction; 50 -75% of cells,
4+ highly positive membrane reaction; more than 75% of cells.

Statistics

Data preparation was performed using a Microsoft Office Excel computer spreadsheet. The data are presented in tabular form. Categorical and nominal values are presented through the appropriate frequencies and proportions, while the differences between them were analyzed by Fisher's exact test, and Fisher-Freeman-Halton test in the case of tables larger than 2x2 format.

Continuous values were shown across the median and interquartile ranges, and the differences between them were analyzed by the Mann-Whitney U test. P values less than 0.05 were considered significant. IBM SPSS Statistics software, version 25.0 (https://www.ibm.com/analytics/spss-statistics-software) was used in the analysis.

RESULTS

Out of 80 analysed tissue samples, 30 were histologically confirmed OLs, 30 histologically confirmed OSCCs and 20 HOM. Differences between groups according to the age and gender are presented in Table 1.

Table 1: Demographics and clinical features of the patients.

		OL N=30				OSCC N=30		HOM N=20		P¹	P²	P³
				Dysplasia								
		N	%	N	%	N	%	N	%			
Gender	Male	15	50,0%	3	10,0%	27	90,0%	10	50,0%	**<0,001**	1,000	**0,003**
	Female	15	50,0%	1	3,3%	3	10,0%	10	50,0%			
Age (years)	Median (IQR)	58,0 (53,0 - 71,0)				66,5 (52,2 - 72,0)		53,5 (49,0 - 61,2)		0,437	0,104	**0,017**

p¹ Fisher's exact test - OL vrs. OSCC
p² Fisher's exact test - OL vrs. HOM
p³ Fisher's exact test - HOM vrs. OSCC
* Mann-Whitney U test

Differences between the groups with respect to the inflammation described on HE preparations are shown in Table 2.

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Table 2: Differences between groups with regard to HE inflammation.

		OL N=30		OSCC N=30		HOM N=20		P[1]	P[2]	P[3]
		N	%	N	%	N	%			
Inflammation HE	No inflammation	3	10,0%	1	3,6%	15	75,0%	0,067	**<0,001**	**<0,001**
	Deficient	17	56,7%	24	85,7%	5	25,0%			
	Moderate	7	23,3%	1	3,6%	0	0,0%			
	Dense	3	10,0%	2	7,1%	0	0,0%			

p[1] Fisher's exact test - OL vrs. OSCC
p[2] Fisher's exact test - OL vrs. HOM
p[3] Fisher's exact test - HOM vrs. OSCC
* Mann-Whitney U test

Labeling index differences of markers in relation to HE inflammation in OL

The inflammatory infiltrate described on HE preparations in the stroma correlated with the expression of Ki67 in the inflammatory infiltrate in the stroma (p = 0.013).

Table 3: Labeling index differences of markers in relation to HE inflammation in OL.

		HE inflammation								p
		No inflammation		Deficient		Moderate		Dense		
		N	%	N	%	N	%	N	%	
CD44 epithelium	<5%	1	33,3%	4	23,5%	2	28,6%	0	0,0%	0,360
	5-25%	1	33,3%	4	23,5%	0	0,0%	0	0,0%	
	25-50%	1	33,3%	6	35,3%	2	28,6%	0	0,0%	
	50-75%	0	0,0%	3	17,6%	2	28,6%	2	66,7%	
	>75%	0	0,0%	0	0,0%	1	14,3%	1	33,3%	
CD44 inflammation	<5%	2	66,7%	4	23,5%	2	28,6%	0	0,0%	0,118
	5-25%	1	33,3%	9	52,9%	0	0,0%	1	333%	
	25-50%	0	0,0%	4	23,5%	3	42,9%	1	33,3%	
	50-75%	0	0,0%	0	0,0%	2	28,6%	1	33,3%	
	>75%	0	0,0%	0	0,0%	0	0,0%	0	0,0%	
Ki67 epithelium	<5%	3	100,0%	5	29,4%	2	28,6%	0	0,0%	0,163
	5-25%	0	0,0%	3	17,6%	4	57,1%	1	33,3%	
	25-50%	0	0,0%	8	47,1%	1	14,3%	2	66,7%	
	50-75%	0	0,0%	1	5,9%	0	0,0%	0	0,0%	
	>75%	0	0,0%	0	0,0%	0	0,0%	0	0,0%	
Ki67 inflammation	<5%	3	100,0%	17	100,0%	5	71,4%	1	33,3%	**0,013**
	5-25%	0	0,0%	0	0,0%	2	28,6%	2	66,7%	
	25-50%	0	0,0%	0	0,0%	0	0,0%	0	0,0%	
	50-75%	0	0,0%	0	0,0%	0	0,0%	0	0,0%	
	>75%	0	0,0%	0	0,0%	0	0,0%	0	0,0%	

Immunohistochemical expression of CD44 and Ki67 in the epithelium of the groups

A significant difference between groups with respect to expression of CD44 and Ki67 in the epithelium were detected (Table 4.).

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Table 4: Expression of CD44 and Ki67 in the epithelium of the groups.

		OL N=30		OSCC N=30		HOM N=20		P¹	P²	P³
		N	%	N	%	N	%			
CD44 epithelium	<5%	7	23,3%	0	0,0%	13	65,0%	**<0,001**	**0,005**	**<0,001**
	5-25%	5	16,7%	0	0,0%	5	25,0%			
	25-50%	9	30,0%	8	26,7%	2	10,0%			
	50-75%	7	23,3%	10	33,3%	0	0,0%			
	>75%	2	6,7%	12	40,0%	0	0,0%			
		OL N=30		OSCC N=30		HOM N=20		P¹	P²	P³
		N	%	N	%	N	%			
Ki67 epithelium	<5%	10	33,3%	0	0,0%	16	80,0%	**<0,001**	**0,001**	**<0,001**
	5-25%	8	26,7%	1	3,3%	4	20,0%			
	25-50%	11	36,7%	17	56,7%	0	0,0%			
	50-75%	1	3,3%	10	33,3%	0	0,0%			
	>75%	0	0,0%	2	6,7%	0	0,0%			

p¹ Fisher's exact test - OL vrs. OSCC
p² Fisher's exact test - OL vrs. HOM
p³ Fisher's exact test - HOM vrs. OSCC
* Mann-Whitney U test

Immunohistochemical expression of CD44 and Ki67 in the inflammatory infiltrate of the groups

A statistically significant difference between the groups with respect to expression of CD44 in the inflammatory infiltrate was detected. Significant difference with respect to the expression of Ki67 in the inflammatory infiltrate between OSCC and OL and OSCC and HOM were detected (Table 5.).

Table 5: Expression of CD44 and Ki67 in inflammatory infiltrate of the groups.

		OL N=30		OSCC N=30		HOM N=20		P¹	P²	P³
		N	%	N	%	N	%			
CD44 Inflammatory infiltrate	<5%	8	26,7%	2	6,7%	18	90,0%	**0,017**	**<0,001**	**<0,001**
	5-25%	11	36,7%	11	36,7%	2	10,0%			
	25-50%	8	26,7%	17	56,7%	0	0,0%			
	50-75%	3	10,0%	0	0,0%	0	0,0%			
	>75%	0	0,0%	0	0,0%	0	0,0%			
		OL N=30		OSCC N=30		HOM N=20		P¹	P²	P³
		N	%	N	%	N	%			
Ki67 Inflammatory infiltrate	<5%	26	86.7%	3	10.0%	20	100.0%	**<0,001**	0.140	**<0,001**
	5-25%	4	13.3%	24	80.0%	0	0.0%			
	25-50%	0	0.0%	3	10.0%	0	0.0%			
	50-75%	0	0.0%	0	0.0%	0	0.0%			
	>75%	0	0.0%	0	0.0%	0	0.0%			

p¹ Fisher's exact test - OL vrs. OSCC
p² Fisher's exact test - OL vrs. HOM
p³ Fisher's exact test - HOM vrs. OSCC
* Mann-Whitney U test

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Positive correlation was observed between HE inflammation and Ki67 in inflammatory tissue of the OL group. Significant and positive correlations were observed with both CD44 in epithelium and inflammatory tissue of the same group. Positive correlation between CD44 in inflammatory infiltrate with CD44 and Ki67 in the epithelium only in OL group were observed (Table 6.).

Table 6: Correlation of CD44 and Ki67 in epithelium and in inflammatory infiltrate with age and HE inflammation in OL and OSCC; Correlation of CD44 and Ki67 in inflammatory infiltrate with CD44 and Ki67 in epithelium.

		OL N=30		OSCC N=30	
		Age (years)	HE inflammation	Age (years)	HE inflammation
CD44 epithelium	Correlation Coefficient	0,314	**0,445**	0,025	-0,294
	P	0,091	**0,014**	0,897	0,129
CD44 Inflammatory infiltrate	Correlation Coefficient	0,127	**0,479**	-0,001	-0,022
	P	0,502	**0,007**	0,994	0,912
Ki67 epithelium	Correlation Coefficient	0,080	0,202	0,122	0,373
	P	0,673	0,284	0,521	0,051
Ki67 Inflammatory infiltrate	Correlation Coefficient	-0,131	**0,556**	0,306	-0,019
	P	0,492	**0,001**	0,100	0,924
		OL N=30		OSCC N=30	
		CD44 inflammatory infiltrate	Ki67 inflammatory infiltrate	CD44 inflammatory infiltrate	Ki67 inflammatory infiltrate
CD44 epithelium	Correlation Coefficient	**0,765**	0,251	0,282	0,165
	P	**<0,001**	0,181	0,132	0,383
Ki67 epithelium	Correlation Coefficient	**0,565**	0,072	0,290	0,351
	P	**0,001**	0,706	0,120	0,057

DISCUSSION

Leukoplakia is one of the most common oral precancerous lesions, which have potential for malignant transformation. Histopathological findings, which shows the presence of dysplasia indicates possibility of malignant transformation. However, some lesions with dysplasia will evolve into OSCC and some not, as well as one without dysplasia [3]. To date, there is no widely accepted reliable marker, which will show which lesion will evolve into OSCC. In the present study, we investigated expression of CD44 and Ki67 in OL.

Out of 30 OL cases, positive immunohistochemical reaction to CD44 in the epithelium were recorded in 23 OL (76.7%) as opposed to HOM where the same reaction was positive in 7 (35%) of total 20 cases. Contradictory results were noted in a study by Venkat Naga et al. [26] where decreased CD44 expression was associated with more severe epithelial dysplasia. From the obtained results, authors concluded that the association between the degree of dysplasia and reduced CD44v6 expression may indicate early cellular changes, which start from physiological cell-cell and cell-extracellular matrix interactions and move towards pathological ones, thus favoring invasion and early development of malignant tumors in the oral cavity [26]. A positive reaction to CD44 in the epithelium was also observed in the OSCC group, with the percentage of positive cells being slightly higher and ranging from 25-75%. The literature data on the value of CD44 as a marker of cancer stem cells (CSC) are contradictory. For this reason, some authors emphasize the need to combine CD44 with other markers because it is assumed that all positive cells are not a pure populations of CSC [27]. In addition, the existence of various isoform variants of CD44, which have common but also individual different functional roles, also contributes to the variability of the results obtained in the conducted research. For example, in some studies, increased immunohistochemical expression of certain isoform variants of the CD44 molecule (CD44v3, CD44v4, CD44v6…) with an advanced stage of the disease and poor survival in patients with OSCC were reported [17]. However, in other studies, a link was found between the reduced expression of these isoform variants

and the advanced stage of the disease and poor survival [28]. Also, in some studies, no association was found between the marker CD44 and the stage and prognosis of the disease [29]. In this study, standard form of CD44 (CD44s) was observed, but not the isoform variants, which can certainly be the reason for the variability of the obtained results when compared to some literature data.

Furthermore, in this study a positive immunohistochemical reaction of CD44 in inflammatory infiltrate in 73.4% of OL cases was recorded, while in HOM this reaction was positive in only 10% of cases ($p < 0.001$). There was also a statistically significant difference in relation to the described inflammation on HE staining preparations in the OL and OSCC in relation to HOM ($p < 0.001$). The presence of inflammation in potentially malignant lesions of the oral cavity may be a factor that will at some point prevail toward the onset of a malignant change. This fact is explained by the well-known theory of the association between chronic inflammation and cancer, as well as the fact that leukocytes and other inflammatory cells, which are responsible for defense against inflammation, also produce reactive oxygenated and nitrogenized radicals that can induce DNA damage. Such successive and multiple damages and regenerations may ultimately result in mutations [30]. Therefore, the detection of CD44 in inflammatory infiltrate can be considered not only an indicator of inflammation, but also in some way a marker which will according to the strength of its expression in inflammation, detect OL with higher risk of malignant transformation. In this study, immunohistochemical expression of CD44 and Ki67 in OL were examined, which has not been recorded in any previous study. Sinanoglu et al [31] reported mean value of labeling index (LI) of 36% in 19 nondysplastic OL. Such OLs carries a significant risk for malignant transformation and such patients are recommended to be monitored, as well as those with dysplasia. This potential for malignancy in histopathologically nondysplastic epithelium may mask a mechanism of carcinogenesis that has not yet developed [32]. In the present study, out of a total of 4 OLs with dysplasia (3 mild and one severe) on the pathohistological finding, negative immunohistochemical reactions of Ki67 and CD44 were detected in one case of mild dysplasia. This can be explained by the well-known assertion from the literature that some leukoplakia with dysplasia will never undergo malignant transformation, while some without the presence of it will progress to oral cancer [33]. Positive immunohistochemical reaction for Ki67 in the epithelium was observed in the majority of OSCC (100%) and OL (66.7%) groups, and in 20% of HOM. The results are consistent with the literature data showing a good prognostic value in oral precancerous lesions and OSCC [34-36]. Increased risk for the development of malignancy may be supported by the result of this study, which showed a positive correlation between inflammatory infiltrate on HE preparations and increased expression of CD44 in the epithelium ($r = 0.445$; $p = 0.014$) of OL. This result confirms the role of chronic inflammation in cancer development [30]. In contrast, negative correlation of CD44 on HE inflammation preparations in OSCC ($r = -0.294$; $p = 0.129$) probably supports the fact that the point of no return has been crossed, which would mean that in this case the inflammatory infiltrate probably no longer plays a role in the further progression of the tumor. Positive correlation between CD44 in inflammatory infiltrate and in epithelium was statistically significant ($r = 0.765$; $p < 0.001$), as well as correlation between CD44 in inflammation and Ki67 in epithelium ($r = 0.565$; $p = 0.001$) of the OL group, but it was not the case in the OSCC ($r = 0.282$, $p = 0.132$; $r = 0.290$, $p = 0.120$). Based on the obtained results, it can be concluded that CD44 has shown to be highly sensitive and specific in the diagnosis of potentially malignant oral lesions such as OL.

Considering results of this study and results of previously published studies, related to the expression of CD44 and Ki67 in the OL and OSCC, we can say that both of these markers play an important role in carcinogenesis of oral mucosa. However, the wide range of molecular aberrations that occur with a relatively high frequency in potentially malignant lesions of the oral mucosa and epithelial dysplasia, then difficulties in terms of standardization and evaluation of markers on small samples, give results that still due to the mentioned limitations failed to be implemented in clinical practice. Therefore, further studies on a larger number of samples that will use standardized immunohistochemical methods to overcome the mentioned limitations are needed.

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