Original Paper

Histopathological assessment of oral leukoplakia. Osteonectin as possible biomarker for further diagnostics

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Clinical evaluation of oral leukoplakia (OL), confirmed by the histological evaluation of the suspected area, provides the gold standard for diagnostics of this pathology. The aim of present study was to encrypt the significance of the histopathological results (oral intraepithelial neoplasia – OIN, WHO 2005, Ljubljana classification systems) of OL. The usefulness of osteonectin as a biomarker of changes in the oral cavity epithelium was evaluated.

IRS Score to evaluate osteonectin (SPARC – secreted protein acidic and rich in cysteine) production in oral mucous tissues was modified, with the aim of adapting the diagnostic measurements to the OL cell environment. In total, 37 formalin-fixed, paraffin-embedded (FFPE) blocks from patients with clinically diagnosed OL, and 29 FFPE blocks from patients with OSCC were evaluated. The OIN and system from Ljubljana were compared, to adjudicate which was most compatible with WHO 2005 histopathological assessment.

Increased production of SPARC was observed, with the progression in severity of pathological changes in the oral mucosa, from simple hyperplasia, through dysplasia, to OSCC. The WHO 2005 and the OIN classification systems can be applied interchangeably.

Key words: osteonectin, SPARC, oral leukoplakia, oral neoplasms.

Introduction

Oral leukoplakia (OL) is classified by the WHO as an oral potentially malignant disorder (OPMD), with the possibility of transformation to oral squamous cell carcinoma (OSCC) in 0.13% to 34% of cases, depending on the source of the data given [1, 2]. Oral leukoplakia, with an average worldwide prevalence of 2%, is a multifactorial disorder. Among the possible risk factors for its development and progression, smoking habit and alcohol abuse, or *Candida albicans* suprainfection, are most commonly mentioned. All those might trigger the genetic alterations leading to OSCC [3, 4, 5, 6]. Relatively little is known about OSCC as a disease affecting patients worldwide, and therefore the disease is still an open chapter of the recent research [6, 7]. Hence, proper diagnostics of OPMD, which leads to the development of OSCC, is of great relevance.

Alcohol consumption might have mutagenic and carcinogenic properties, because of toxic ethanol metabolite production – acetaldehyde – and its action on the oral mucous membrane. A lot of research was conducted to find the main cause for the development of these pathologies, but there is still a little evidence of molecular changes leading to disease progression. This condition varies from one geographical area to another, although it was found that the higher risk of neoplastic transformation begins in anatomical areas of the oral cavity covered with non-keratinised mucosa [8, 9, 10, 11].

Proper diagnosis of OPMD relies on the patient's medical history, clinical assessment, and histopathological diagnosis of oral epithelium changes [12]. According to the World Health Organisation (WHO), histopathological evaluation remains the gold standard for OL diagnostics [13]. After histopathological diagnostics of the cell progression, the clinical treatment schedule might be chosen, when the potential presence, or grade of dysplasia is considered [12], but the evaluation systems used by pathologists differ between laboratories. Changes in the field of results presented by pathologists influence the possibility of proper clinical evaluation of the disease, because of non-coherent names of grades between classification systems. Proper understanding of classification systems grades, are crucial for the clinical proceedings after the histopathological evaluation. In this article the conformity of two classification systems – the OIN system and the Ljubljana system – were compared with the WHO 2005 system [14] (Table I). All listed classification systems are used interchangeably, and the use of a specific system depends on the pathomorphologists' preferences or regional requirements for histopathological reporting. The WHO classification system is considered as a gold standard for histological evaluation and is updated frequently. The latest update, WHO 2017, provided the extension of mild dysplasia grade to the grade previously called "squamous hyperplasia" in the WHO 2005 classification [14, 15]. Because such a change might be of great importance for proper treatment of patients, it shows the need for unification of the classification systems of oral changes, and for the search for modern biomarkers reflecting the actual state of the patient's disease.

Secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin and BM-40 (basement membrane protein 40) is a phosphorylated glycoprotein with a molecular weight from 32 to 43 kDa [16, 17, 18]. The presence of this protein has been demonstrated in tissues that undergo frequent renewal, such as chondral tissue or the epithelium of the gastrointestinal tract. In addition, this protein appears in endothelial cells in response to damage, as well as in regions of active bone remodeling [18], and is observed in patients with periodontitis during periodontal

Table I. Comparison between WHO systems from 2005, Ljubljana System, and OIN system

Oral intraepithelial neoplasia (OIN)	WHO, 2005	Lublana (SIL)
n/a	Squamous cell hyperplasia	Simple squamous cell hyperplasia
OIN 1	Mild dysplasia	Basal/parabasal cell hyperplasia
OIN 2	Moderate dysplasia	Atypical hyperplasia
OIN 3	Severe dysplasia	Atypical hyperplasia
	Carcinoma *in situ*	Carcinoma *in situ*

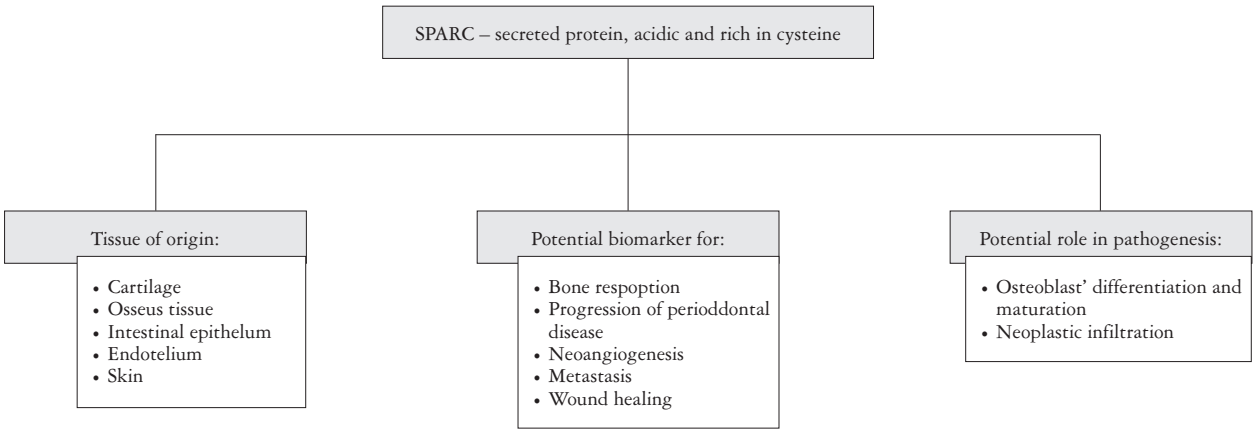

Fig. 1. Characteristics of SPARC protein

repair processes [19]. Figure 1 presents a summary of SPARC's characteristics. It is suggested that osteonectin may participate in cancer progression. Protein activity causes increased metalloproteinase-2 (MMP-2) activity in human tumour tissues, contributing to the development of neoangiogenesis [18]. The specific function of SPARC in neoplastic processes in OSCC has not yet been thoroughly described, and it is most important that it be discovered, because in other types of cancer cells its function may have a stimulatory or inhibitory effect on the malignancy process [17]. It has been suggested that in the processes of malignant oral epithelium transformation, the processes of increased tissue remodelling may be involved, intensified under the influence of various repair processes stimuli. This leads to the conclusion that a possible role of SPARC is as an indicative progression marker in oral pathologies. When we search for such keywords as "SPARC AND oral leukoplakia" or "osteonectin AND oral leukoplakia" in the medical search engine – PubMed.gov – there is only one publication available in the results. Therefore, the basis for the use of this molecular marker seems open to discussion. In this study, the expression of osteonectin in the precancerous state of OL (along with the division into particular histopathological stages of oral mucous biopsies) was evaluated in 31 cases, and in 29 cases of OSCC.

Material and methods

Tissue specimens and patients' medical history

Formalin-fixed, paraffin-embedded (FFPE) blocks with biopsies from the oral mucous membrane were obtained from the archives of the Department of Pathomorphology of a local hospital. Formalin-fixed paraffin-embedded blocks prepared from biopsies taken from patients of the Oral Pathology Department, with clinical suspicion of OL, were afterwards processed for histopathology. The leading pathologist re-diagnosed FFPE samples with the use of three different classification systems – Oral Intraepithelial Neoplasia (OIN), the classification system from Ljubljana, and the WHO 2005.

Patients' clinical charts from the Department and Clinic of Maxillofacial Surgery of the local hospital were analysed with respect to all compliance acts and the anonymity of all individuals. The approval of the local Ethics Committee was obtained for the research.

Immunohistochemical staining

Tissue material consisted of archived oral mucosa biopsies fixed in 10% buffered formalin and paraffin-embedded:

- 37 blocks from 31 different patients from diverse areas of OL,
- 29 blocks from 29 patients with oral neoplasms.

From the prepared FFPE blocks, the samples of 5 μm thickness were obtained, with the use of microtome apparatus. Afterwards, deparaffinization in xylene was carried out, and material was put on SuperFrost slides (Super Frost Plus Menzel GLASSER, Braunschweig, Germany). For immunohistochemistry reaction SPARC Mouse Monoclonal Antibody was used (AON-5031, No.sc-73472, Santa Cruz Biotechnology, US). The described process was carried out with the use of Target Retrival Solution and PT Link Rinse Station at pH 9 (temp. 97ºC, 20 min). The samples were washed in TBS and incubated with original antibody in an automated IHC Dako Link 48 Autostainer system (room temperature, 20 min). VVisualisation was proceeded in EnVison FLEX (DakoCytomation) reagent, and samples were dyed in haematoxylin, according to the guidelines of the manufacturers. The primary staining was accompanied by a negative control.

Evaluation of SPARC protein expression

Production of SPARC protein by immunohistochemical reaction was evaluated in various stages of oral mucous membrane pathologies – from OL, in comparison with SPARC production in OSCC. The negative control was the non-invaded margin of the oral epithelium of the oral mucous membrane. Expression of SPARC protein was evaluated in Yamada's semi-quantitative scale of immunoreactivity (Immunoreactive Score – IRS) [17] in the author's modification, based on Remmele's immunoreactivity scale [20]. In the evaluation of SPARC expression, two parameters were considered together: the percentage of cells showing positive cytoplasmic reaction and the intensity of colour reaction.

Definitive rating of immunohistochemical reaction was included in a five-grade semi quantitative modified IRS scale, for the purpose of this publication referred to as the IRS Score. The described parameters of immunohistochemical evaluation are presented in Table II.

Table II. ImmunoreactiveYamada Score, IRS in the author's modification

Percentage of cells with colour reaction	Reaction	Score
No cells, or number of cells under < 5%	Negative	0
5-25% positive cell reaction	Weak	1
26-50% positive cell reaction	Moderate	2
51-80% positive cell reaction	Strong	3
> 80% positive cell reaction	Very strong	4

Table III. Characteristics of patients

	OSCC	Leukoplakia
Number of patients	29	31
Sex		
Men/Women	59%/41%	61%/39%
Average age	65.1	57.5
Smoking habit:	41%	26%
Patients who gave up smoking during the treatment	17%	6%
Patients smoking more than 10 cigaretts per day	21%	16%
Previous oncological treatment in oral cavity area	17%	13%
Alcohol habit – patient's declaration	24%	6%
General health problems		
Diabetis	14%	10%
Hipertension	34%	32%
Varicose veins of the lower limbs	24%	0
Gastroenterological disorders	21%	6.5%

Table IV. Localisation of leukoplakia and squamous cell carcioma changes in oral mucosa

		Localisation							Total
		Tongue	Gingiva	Lip	Cheek	Angle of the mouth	Hard palate	The bottom of the mouth	
Number	OSCC	10	9	0	4	0	0	2	25
%		40.00%	36.00%	0.00%	16.00%	0.00%	0.00%	8.00%	
Number	Leuko-plakia	4	6	3	10	1	3	2	29
%		13.79%	20.69%	10.34%	34.48%	3.45%	10.34%	6.90%	

Results

Patients' characteristics

A statistically significantly higher average age of patients with OSCC was found in comparison to patients with OL ($p = 0.0150$). Patients general characteristics and medical history results are presented in Table III. Referring to the location of the lesion, a slightly more frequent occurrence of OL has been demonstrated on the buccal mucosa and for OSCC on the tongue of patients. The results of the total percentage of localizations in the oral cavity of both pathologies are shown in Table IV.

Histopathological evaluation

Oral neoplasms group

In the group of 29 oral neoplasms, according to the WHO classification, the following were identified:

- 13 biopsies of *Carcinoma planoepithelialekeratodes*,
- 12 biopsies of *Carcinoma planoepithelialeakeratodes,*
- 4 biopsies of *Carcinoma verrucosum.*

Among the *C. planoepithelialekeratodes* and *akeratodes*, the grade of histopathological malignancy (Grading, G) was evaluated as follows:

- 8 G1 cancers in the highest degree of differentiation (the lowest grade of malignancy),
- 14 G2 cancers in the middle degree of differentiation (the moderate grade of malignancy),
- 4 G3 cancers with the lowest degree of differentiation (the highest degree of malignancy).

Oral leukoplakia group

This section compares the results of the comparative histopathological assessment of oral mucous biopsies with the use of two different histological systems – Oral Intraepithelial Neoplasia (OIN) (Table VI) and the system of grading from Ljubljana (Table VII) – in reference to the WHO 2005 system.

The Wilcoxon statistical test was used in the study. Comparison of the results obtained by

Table V. Evaluation of the results for SPARC expression in oral leukoplakia and oral squamous cell carcinoma

	GROUP	SPARC 0	SPARC1	SPARC 2	SPARC 3	SPARC 4
Numeric value	OSCC	3	9	8	4	2
%		11.54%	34.62%	30.77%	15.38%	7.69%
Numeric value	Leukoplakia	20	9	1	0	0
%		66.67%	30.00%	3.33%	0.00%	0.00%

Table VI. Comparison of histopathological results obtained after applying the WHO classification in comparison with the OIN system

		OIN			
		0	1	2	3
WHO	0	17	2	0	0
	1	0	4	0	0
	2	0	0	3	0
	3	0	0	0	1

Table VII. Comparison of histopathological results obtained after applying the WHO classification compared to the system from Ljubljana

		Ljubljana			
		0	1	2	3
WHO	0	15	2	0	0
	1	0	2	2	0
	2	0	0	3	0
	3	0	0	0	1

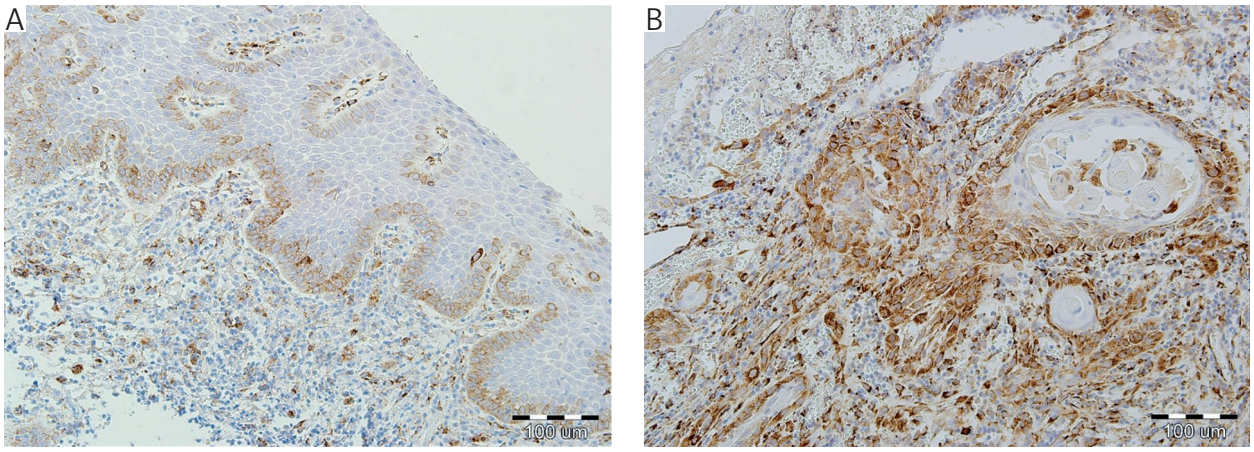

Figure 2. Image A) represents simple hyperplasia of the oral mucosa epithelium. IHC reaction with SPARC antibody Score 1. Image B) represents squamous cell carcinoma G1. IHC reaction with SPARC antibody – Score 3

histopathological analysis of the material showed that the OIN system was the most consistent with the WHO classification system, with a coefficient of $p = 0.180$.

SPARC immunohistochemical staining

Expression of SPARC protein with the use of immunohistochemistry was evaluated in the case of 26 patients with diagnosed oral neoplasms. Lack of expression (SCORE 0) was evaluated in 11.54% of patients, SCORE 1 expression was evaluated in 34.63%, SCORE 2 expression was evaluated in 30.77%, SCORE 3 expression was evaluated in 15.38%, and the 4th grade of expression – SCORE 4 – was evaluated in 7.69% of all patients.

Expression of SPARC protein with the use of immunohistochemistry was evaluated in 30 patients with clinically diagnosed leukoplakia. Lack of expression (SCORE 0) was evaluated in 66.67%, SCORE 1 (Fig. 2) expression was evaluated in 30.00%, SCORE 2 expression was evaluated in 1 case, and SCORE 3 (Fig. 2) and 4 were not present in the whole group of OL. All the described results are shown in Table V.

The Spearman test was used for statistical evaluation of all obtained results. With the progression of severity of the oral mucosa pathological changes (from mucosal hyperplasia to *C. planoepitheliale*), increased production of osteonectin was observed. Thus, a correlation was found in the production

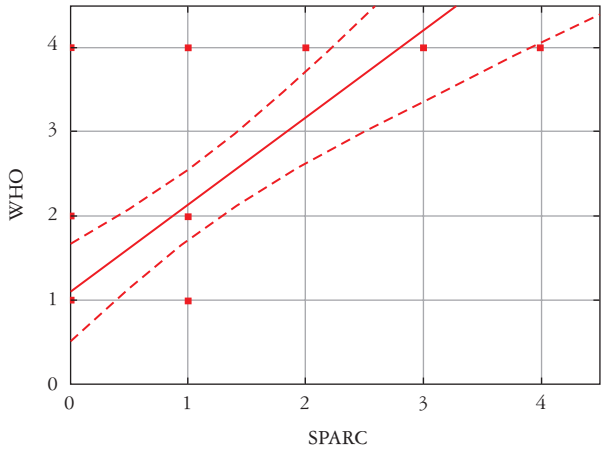

Fig. 3. Correlation between SPARC protein production and stages of oral mucous progression

of SPARC protein among oral mucous pathologies. This correlation is shown in Fig. 3.

Discussion

Leukoplakia is the most common oral, potentially malignant, disorder, with prevalence of 4.11% based of the meta-analysis of Mello *et al*. [12] Taking into consideration the patients' gender, in the present study men were more often affected by OL than women, and represented 61% of all patients with clinically diagnosed OL. According to Shearston *et al*., this premalignant lesion more often affects smokers, and usually shows its first clinical symptoms in the 4th decade of life [2]. The average age of patients with OL from the Lower Silesia region was 57.5 years, and that places the results of this research in the first standard deviation of the results obtained by Ang *et al.* [6] and in line with those of Shearston *et al*. [2]. In total 26% of patients from the OL group of the presented research declared having a smoking habit. Common sites of OL include the tongue and buccal mucosa [2], and in the present study the most common site of OL was buccal mucosa.

OSCC is ranked as the eighth most common cancer in males worldwide, but in females it is not on the list of top ten cancers [7]. From all patients affected with OSCC in the present research, men represented 59%. According to the Polish National Cancer Registry, malignant neoplasms of the tongue are rare and constitute about 0.5% incidence of all reported malignant neoplasm cases among men in Poland, and about 0.2% of all reported malignant neoplasms cases among women [21]. In the present research, this localisation of OSCC was most frequent in all patients, reaching 40% of all analysed OSCC cases. The highest incidence of tongue cancer occurs in patients during the sixth to seventh decade of life, according to the Polish National Cancer Registry [21]. In our research the average age of all patients with OSCC was 65.1 years.

Histopathological reporting of OL is subjective and external quality assurance is crucial here [22]. This study provided evaluation of two different classification systems – OIN and Ljubljana – both used by pathologists during histopathological evaluation of oral biopsies. The aim was to compare both classification systems with the WHO 2005 system. Ljubljana classification is considered to be an alternative OPMD assessment, but it was developed for use in laryngeal pathologies [22]. For that reason, the term "dysplasia" in not present, and the term "atypical hyperplasia" is used instead [22]. This change has to be taken into consideration when the results are received, because of the significance of dysplasia diagnostics in further clinical proceedings. In the present article the conclusion was reached that the highest coherence with the WHO 2005 system was received by the use of OIN classification. Oral intraepithelial neoplasia assessment is based on the Squamous Intraepithelial Neoplasia/dysplasia (SIN/dysplasia) classification from 2005, first created for the uterine cervix diagnostics, and afterwards extended to other mucosae [14, 23]. The OIN system grades lesions as high and low grade, and concordance with the WHO 2005 system presented in this paper might show the reasoning for the cut-off point for dysplasia in oral mucous membrane samples [14].

Given the latest available data, regarding the possibility of malignant transformation, OL might be a pathological condition of medium transformation. Shearston *et al.* showed that this incidence in the Australian population was 1.49%, with an average time of malignant transformation of 5.2 years [2]. The term "oral leukoplakia" might be used to cover clinical changes in the oral cavity, but confirmation in the histopathological results is needed, and therefore collaboration between a pathologist and a dentist is crucial [24]. Proper diagnostics should lead to histopathological assessment of changed oral mucosa to distinguish the grade and presence of dysplasia by evaluation of FFPE [24]. As many researchers show, traditional histopathological diagnostics of OL might not be enough to identify all lesions that possibly might be undergoing malignant transformation process. Therefore, there is a need of new histopathological and molecular biomarkers, that could be used to detect this process with greater specificity [2]. The function of osteonectin as a key regulator is to control the cell proliferation, migration, and survival. Despite growing interest in the role of SPARC in various types of cancer, information on protein involvement in the issue of tumor formation and progression is contradictory [16]. Studies by many authors prove the relationship of SPARC protein with the malignancy of breast cancer, melanoma, osteosar-

coma, glioblastomas and bladder cancer. On the other hand, the inhibitory effect of the glycoprotein on the development of ovarian cancer has been demonstrated, as well as the use of SPARC in the prognosis of pancreatic adenocarcinoma or colon cancer [16, 17, 18]. Osteonectin as a predictive marker in dentistry is still a matter of research. Animal models are used to broaden the understanding of the role of SPARC in periodontitis [19] and its association with cancerisation of oral squamous epithelium, as discussed in [17], along with research performed in order to evaluate the correlation between SPARC expression in tumour cells in OSCC with the worst pattern of invasion [25]. Demonstration of whether the aforementioned dependence could be used in the progression of leukoplakia-type neoplastic states was one of the aims of this study.

It was confirmed that SPARC may be an indicator of mutations during the initial stages of epithelial carcinogenesis, however, the usefulness of this biomarker ends at this stage, and it was not indicated as a predictive factor in cancer prognosis [17]. The described research of Yamada *et al*. led the authors of the present study to diagnose the expression of SPARC protein in primary stages of oral cell pathology progression in order, to evaluate its possible role as a predictive marker.

During the process of the research presented herein, a higher expression of SPARC was demonstrated in the form of pathological changes in the oral mucosa, which progressed from different histopathological cell stages in biopsies from OL to OSCC, compared to the healthy tissue margins. Based on this study it may be concluded that osteonectin production is induced by dysplastic cells in the initial stages of neoplastic development, it stopped at this stage and was not an indicator of malignancy of oral neoplasms. In order to clearly determine this issue, further studies should be carried out on larger groups of patients.

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The authors declare no conflicts of interest.

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