

Histopathological Classifications of Oral Leukoplakia and its Relation to Cell Proliferative Activity: A Case Series

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ABSTRACT

Aim: This study relates the average number of nucleolar organizing regions (NORs) obtained in a series of cases of oral leukoplakia, with three methods of histopathological classification of such lesion.

Materials and methods: This is a histopathological–histochemical laboratory cross-sectional study. The 18 cases of leukoplakia analyzed were filed at Pathology Service of the Biological Sciences Institute of the University of Passo Fundo, Rio Grande do Sul, Brazil (SDH/ICB/UPF) (2017 and 2018), from which epidemiological data were extracted. New histological sections were performed for impregnation by the argyrophilic nucleolar organizer regions (AgNOR) technique. The histopathological slides were analyzed by photon microscopy (1,000×), and the nuclei of 100 epithelial cells were photographed to count the number of NORs. Three methods were used for the lesions' histopathological classification [World Health Organization (WHO), Brothwell, and binary system]. The means of NORs were compared with the three histopathological classifications by means of the *t* or analysis of variance (ANOVA) statistical tests, at a significance level of 5%.

Results: According to the WHO classification method, most cases (11–61.1%) had a moderate classification. Evaluations by the Brothwell method showed moderate and mild classification in 50 and 38.9% of cases, respectively. According to the binary system, most cases (10–55.6%) had low risk. The average NORs found in 100 nuclei of each of the 18 lesions ranged from 2 to 4. When crossing the average NORs with the histopathological classification methods of the lesions by means of the *t* test or ANOVA, no significant relationship was noted.

Conclusion: The average of NORs is not associated with the histological classifications of leukoplakias. Thus, the AgNOR method should be used with caution when differentiating different histological grades of leukoplakias.

Clinical significance: The AgNOR method should be used with caution to determine the clinical treatment of oral leukoplakias, since no agreement was observed between this method and the histopathological classifications available for such lesion.

Keywords: Argyrophilic nucleolar organizer regions, Diagnosis, Histological classification, Leukoplakia, Prognosis.

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INTRODUCTION

Epidermoid carcinoma is the most common neoplasm that affects the mouth, representing about 90% of the malignancies of such anatomical location.¹ Clinically, in its premalignant stage, epidermoid carcinomas may present as leukoplakias, erythroplasias, or erythroleukoplakias.^{2–6}

The term “leukoplakia” is a clinical name that characterizes the lesion as a predominantly white patch or plaque of the oral mucosa, not scraping removable, and that cannot be clinically classified as any other pathological entity.² It may have a smooth, rough, or warty surface,⁷ occurring mainly in the jugal mucosa and lips, in addition to the alveolar mucosa, tongue, hard palate, soft palate, floor of the mouth, and gingiva.⁶ Histopathological changes in leukoplakia may range from hyperkeratosis and acanthosis to epithelial dysplasia, or even *in situ* carcinoma or invasive squamous cell carcinoma.^{2–4,8}

Worldwide, oral leukoplakia prevalence varies, and it may represent from 11.1 to 48.9% of cancerous changes.^{3,9–12} It is considered a multifactorial etiopathogenesis lesion,¹³ mainly caused by tobacco and alcohol consumption, over 40 age, poor oral hygiene, poor prostheses fitting, and inadvertent sun exposure.^{11,12,14}

Mortality caused by carcinomas correlates with the stage of diagnosis and prevention of cancerous lesions such as oral leukoplakias.^{2,4,15}

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To determine the degree of leukoplakia epithelial dysplasia, classification criteria were created. The most widely used histopathological classification model was proposed by the WHO and evaluates cytological changes and the cellular architectural arrangement of the lesions, determining them as having “mild epithelial dysplasia,” “moderate epithelial dysplasia,” and “severe epithelial dysplasia.”¹⁶ Such model provides information on how much the lesion is prone to malignancy.^{8,14,17}

Nucleolar organizing regions are structures present in the nucleolus of the interphase cells and in the acrocentric

chromosomes pairs 13, 14, 15, 21, and 22 during human cell division.¹⁸ Previous studies have applied the AgNOR method to cancerous lesions and malignant tumors, describing that this technique can be considered a marker for cell proliferation rate and can be used for prognostic purposes.^{12,19,20}

Thus, the present study aimed to correlate the average number of NORs obtained in 100 epithelial cell nuclei of a series of oral leukoplakias with three methods of histopathological classification of such lesion, already recognized in the literature.

MATERIALS AND METHODS

Study Design and Data Collection

The study was approved by the Research Ethics Committee of the University of Passo Fundo (Opinion No. 172/2011) under C. A. A. E. 0034.0.398.000-11. A cross-sectional laboratory histopathological-histochemical study was performed in a series of oral leukoplakia cases. The analyzed cases were filed at the Pathology Service of the SDH/ICB/UPF.

Sample Selection

All cases of leukoplakia diagnosed at SDH/ICB/UPF from 2017 to 2018 were selected. For this purpose, we used histopathological sections (5 µm) of such cases from incisional or excisional oral biopsies performed by dentists from Passo Fundo/RS/Brazil region. The histopathological diagnoses were blindly reviewed and confirmed by an experienced oral pathologist and subsequently compared with the clinical information sent by the dentists. It resulted in a selection of 18 cases of oral leukoplakia.

Inclusion Criteria

The study included all cases histopathologically diagnosed as hyperparakeratosis, hyperorthokeratosis with or without acanthosis, with or without epithelial cell dysplasia; and cases that, in addition to the histopathological features described above, were clinically represented by keratotic white or gray spot or plaque. It was included in the sample only cases with acceptable diagnostic quality, intraoral or lip location, referred by dentists and with information on age, gender, and lesion location.

Exclusion Criteria

We excluded from the sample cases that had insufficient histopathological material for analysis and cases with incomplete or insufficient clinical information to reach the diagnosis of leukoplakia.

Training and Examinations

At this stage of the study, a single dentist (oral pathologist) received calibration training for diagnosis and classification of oral cancerous lesions compatible with leukoplakia. For this step, a set of training blades was applied to the lesions.

Histopathological Classifications

The data were retrospectively collected, since the cases already had a report. Thus, the histopathological diagnosis was confirmed and the histopathological classifications were assigned as follows. All analyzes were performed under the examiner's blinding, through photonic microscopy, with immersion objective (1,000×). Methods were used for histopathological classification of lesions, following the literature.^{21,22}

The WHO method codified the tissue samples into 1, 2, and 3, respectively: (1) "Mild dysplasia": increased number of cells in the basal and parabasal epithelial regions showing nuclear hyperchromatism and pleomorphism; (2) "Moderate dysplasia": bulbous straight processes with increased cell numbers showing nuclear hyperchromatism and pleomorphism, extending to and including basal, parabasal, and spinous layers; (3) "Severe dysplasia": bulbous straight processes with increased cell numbers showing nuclear hyperchromatism and pleomorphism throughout the epithelial thickness.²³ In addition to the previously described classifications for the WHO method, the Brothwell system considered the "carcinoma *in situ*" level, characterized by epithelial alterations showing nuclear hyperchromatism and pleomorphism, which covers the entire thickness of the epithelium, suggesting an early superficial tissue invasion, but without convincing evidence.²¹

The binary system considered: "high risk"—with potential susceptibility to malignant transformation, resulting in at least four architectural alterations and five cytological alterations; and "low risk"—without susceptibility to malignant transformation, being associated with less than four architectural changes or less than five cytological changes.²²

Histochemical Analysis

In addition to the hematoxylin-eosin-stained tissue samples that allowed the diagnosis and histopathological classification of leukoplakia, new tissue sections were obtained from each case (3 µm thick), to be impregnated by the AgNOR technique, described by Ploton et al.,²³ with some modifications:

This technique is described as follows: (1) 50 mL of Mili-Q water was added as an initial solution, adding 0.5 mL of formic acid and 1 g of gelatin. (2) The obtained solution was kept in an oven at 45°C for 1 hour incubation period. Then a second solution was produced with 10 mL of distilled water and 5 g of silver nitrate. (3) Thereby, both solutions were mixed and dripped onto the cell material disposed on the slide, thus remaining for 30 minutes in an oven at 45°C. (4) Finally, a final wash was performed. (5) Such slides were analyzed under microscopy, and the nuclei of 100 epithelial cells were photographed to count the number of NORs per lesion.

Statistical Analysis

Epidemiological data regarding patients and their lesions were obtained from the histopathological reports of the cases for tabulation in Excel™ spreadsheet. They were later submitted to statistical analysis using SSPS software version 23.0 (Chicago, USA). The average number of NORs was compared with the three histopathological classifications through *t* test or ANOVA means, with a significance level of 5% (Fig. 1).

RESULTS

Of 18 analyzed lesions, 61.1% (11 cases) corresponded to males. The age of patients with oral leukoplakia ranged from 25 to 80 years, with a higher frequency between 46 years and 60 years (44.4%) (mean age 53.22 years). Regarding ethnicity, there was a predominance of Caucasians (11 cases, 61.1%), followed by melanoderms (5 cases, 27.8%) and faioderms (2 cases, 11.1%).

Figure 2A describes the information regarding the possible etiological factors of leukoplakias collected in histopathological reports. Table 1 reports additional data regarding cases of oral leukoplakia. Figure 2B represents the average number of NORs

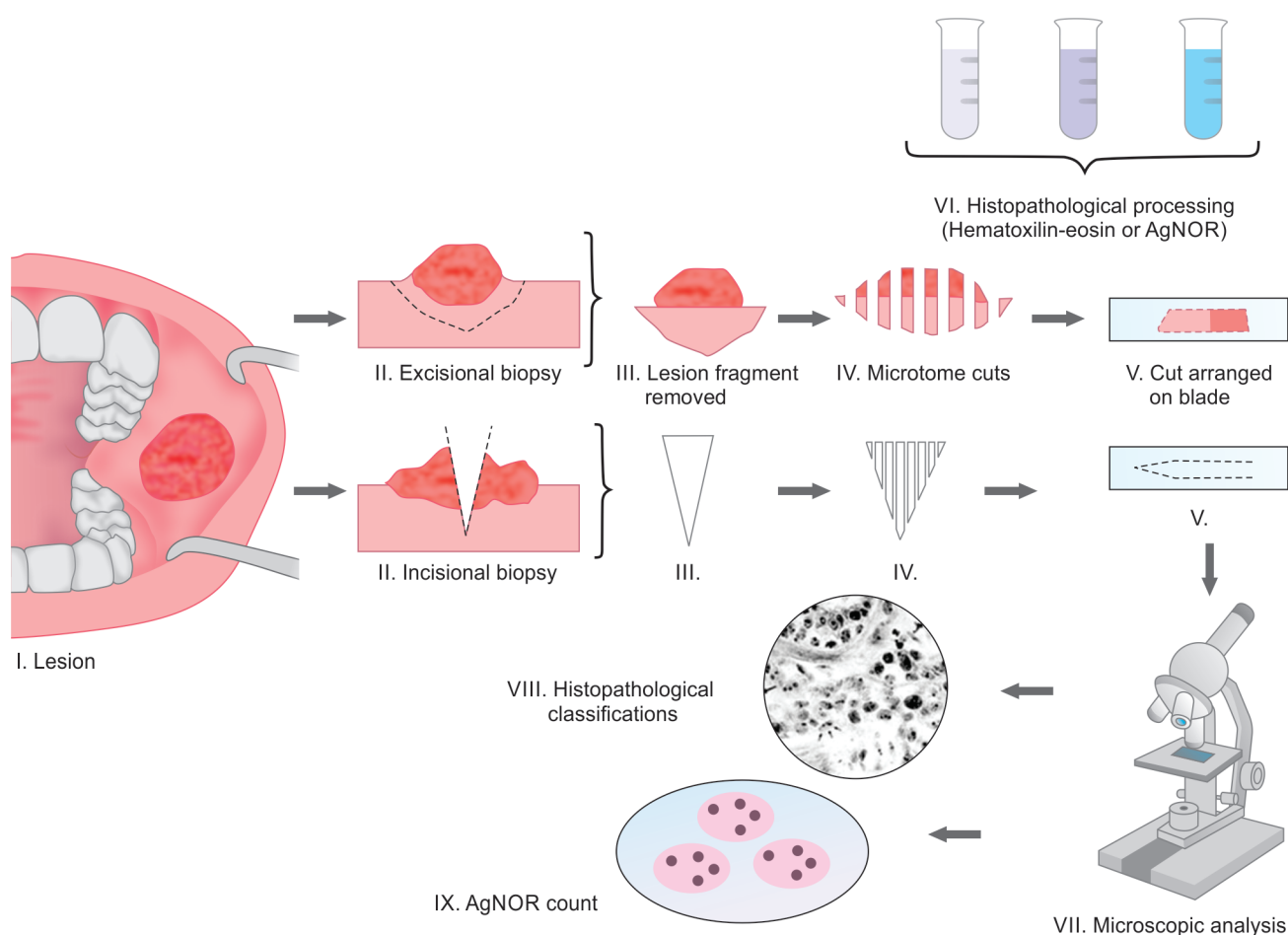
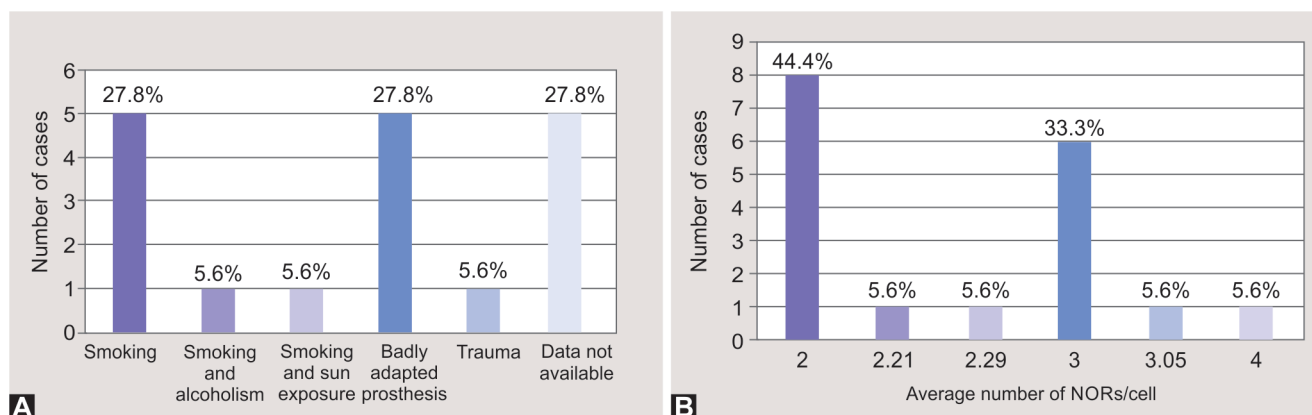


Fig. 1: Study's experimental flow diagram



Figs 2A and B: (A) Distribution of oral leukoplakia cases according to etiological factors; (B) Distribution of NOR average observed in oral leukoplakia lesions

obtained from 100 epithelial cell nuclei from each of the 18 lesions analyzed.

All the three histopathological classification systems for leukoplakia (WHO, binary system, and Brothwell's) were compared with AgNOR results. Correlating the average number of NORs evaluated in 100 leukoplakic epithelial cell nuclei with the methods of histopathological classification of such lesions

proposed by the WHO and the binary system, a statistical *t* test (significance level of 5%) was used. No statistically significant relationship was noted ($p = 0.190$ and $p = 0.542$, respectively) (Tables 2A and B). When the mean number of leukoplakia NORs was related to Brothwell's histological classification, using the ANOVA test means, there was no statistically significant difference ($p = 0.203$) (Table 2C).

Table 1: Absolute and percentage distribution of leukoplakia cases according to clinical features and methods of histopathological classification

Feature	Incidence	Percentage
Location		
Lip	1	5.6
Tongue	2	11.1
Cheek mucosa	6	33.3
Cheek mucosa and alveolar ridge	1	5.6
Hard and soft palate	1	5.6
Alveolar ridge	2	11.1
Alveolar ridge and retromolar area	3	16.7
Alveolar ridge and hard palate	1	5.6
Data not available	1	5.6
Symptomatology		
Absent	6	33.3
Present	5	27.8
Present with burning	3	16.7
Data not available	4	22.2
Fundamental lesion		
Spot	1	5.6
Board	16	88.9
Data not available	1	5.6
Evolution time		
0.5–12 months	6	33.3
13–24 months	2	11.1
25–36 months	6	33.3
Data not available	4	22.2
Size		
≥20 mm	10	55.6
≤20 mm	4	22.2
Data not available	4	22.2
Consistency		
Firm	8	44.4
Firm and elastic	1	5.6
Soft	3	16.7
Soft and firm	3	16.7
Data not available	3	16.7
Borders		
Defined	5	27.8
Undefined	6	33.3
Data not available	7	38.9
Surface		
Flat	5	27.8
Flat and ulcerated	4	22.2
Rough	4	22.2
Rough and ulcerated	1	5.6
Data not available	4	22.2
Coloring		
White (clear)	9	50.0
Reddish white	6	33.3
Black (blackened)	2	11.1
Data not available	1	5.6

Contd...

Contd...

Feature	Incidence	Percentage
Method: WHO		
Mild epithelial dysplasia	7	38.9
Moderate epithelial dysplasia	11	61.1
Method: Brothwell		
Mild epithelial dysplasia	7	38.9
Moderate epithelial dysplasia	9	50.0
Severe epithelial dysplasia	1	5.6
Carcinoma <i>in situ</i>	1	5.6
Method: binary system		
High risk	8	44.4
Low risk	10	55.6
Total	18	100

DISCUSSION

In order to validate the AgNOR method as a determinant tool for oral leukoplakia prognosis, the present study has correlated the average number of NORs with data related to histopathological classifications of leukoplakias.

Several classification systems of epithelial dysplasias have been created. An example is the degree of dysplasias idealized by Brothwell et al.,²¹ which consists in a point-scale classification system. Such system determines lesions as "mild," "moderate," "severe," and "carcinoma *in situ*."¹⁶ This form of classification is very similar to the WHO one, which considers epithelial changes at the architectural level and classifies them based on epithelial stratification. The binary classification system gathers lesions into fewer categories, classifying them only as "high risk" and "low risk" lesions.^{16,22}

Biological markers have been used to determine the behavior of lesions, which should be able to provide information about the biological characteristics involved in cell proliferation or neoplastic cell transformation.^{18–20} One of these markers is the histochemical technique of silver tissue impregnation AgNORs.¹⁹ This technique detects proteins associated with the transcriptional activity of NORs through colloidal silver impregnation, named AgNOR proteins. In this technique, NORs can be visualized under photonic microscopy as small, well-defined dark spots within the cell nucleus.^{19,21}

According to the carried out studies, a high prevalence of leukoplakias was observed in men, which is consistent with the results of the present study, since lesions in men represented 61.1% of the sample.^{12,24} Hallikeri et al.¹¹ observed that 98.78% of the leukoplakia cases they have studied occurred in smokers, whereas in our study, smokers represented only 39.0%. According to Lee et al.,²⁴ the most frequent anatomical location of leukoplakias was the cheek mucosa, which matches with the present study, where such anatomical site represented 33.3% of the cases. Regarding the age of patients, there was a higher prevalence of leukoplakias between the fourth and sixth decades of life. This finding coincides with the statement that oral leukoplakia is most commonly found in individuals over 50 years old.^{10,25}

NORs count is used as a biological marker to estimate the degree of cell proliferation.¹⁹ In this sense, Madan et al.²⁶ demonstrated the difference in the average number of AgNORs between non-dysplastic epithelium and oral squamous cell carcinoma. In another study, the AgNOR count technique helped distinguishing cancerizable lesions and oral squamous cell carcinomas.¹⁹

Tables 2A to C: Statistical crossovers between histopathological classification method proposed by (A) WHO, (B) binary system, (C) Brothwell's histopathological classification method, and average number of NORs

(A)	WHO method	n	Mean	Standard deviation	Standard error
NORs average in 100 nuclei	Mild epithelial dysplasia	7	2.2929	0.50036	0.18912
	Moderate epithelial dysplasia	11	2.6818	0.63361	0.19104
<i>p</i> value = 0.190 (t test)					
(B)	Binary system	n	Mean	Standard deviation	Standard error
NOR average in 100 nuclei	High risk	8	2.6313	0.52300	0.18491
	Low risk	10	2.4500	0.67521	0.21352
<i>p</i> value = 0.542 (t test)					
(C)	Brothwell's classification	n	Mean	Standard deviation	Standard error
NOR mean/100 nuclei	Mild dysplasia	7	2.8871	0.64999	0.24567
	Moderate dysplasia	9	2.3711	0.49334	0.16445
	Severe dysplasia	1	2.0000		
	Carcinoma <i>in situ</i>	1	2.0000		
	Total	18	2.5306	0.60215	0.14193
<i>p</i> value = 0.203 (ANOVA)					

In addition, previous research results suggest that the average of AgNORs may be proportional to the epithelial cell proliferative activity.¹⁹ Furthermore, studies suggest that with the malignancy of the lesion, the number of AgNORs tends to increase.²⁷ Thus, it is hypothesized that the number of NORs of oral leukoplakias would be directly proportional to the increase in their histopathological grading.

Sowmya et al.²⁸ investigated, through the AgNOR method in exfoliative cytologies, the cell proliferative activity of healthy patients with oral leukoplakia, oral submucosal fibrosis, and squamous cell carcinoma. These authors concluded that AgNOR is a simple, sensitive, and inexpensive method for differentiating premalignant from malignant lesions and can be used in conjunction with routine cytomorphological evaluation.

However, in the present study, no statistically significant relationship was observed between the average number of NORs and the histopathological classification methods, i.e., the grading increase of three histological classifications studied was not related to a higher average number of NORs per cell nucleus. The fact that in the present study most of the cases analyzed presented mild or moderate epithelial dysplasia may explain why the NOR count was not useful in differentiating the histological grades of leukoplakias, since the literature states that such technique is more effective in lesions with severe dysplasia.^{19,29}

Spolidorio et al.³⁰ also reported that there were no statistically significant differences in AgNOR count between lesions with different histological grades, only pointing to a morphology and distribution patterns of NORs within cell nuclei distinctions.

The findings of the present study are also supported by results of Carmo and Silva,³¹ who studied 22 cases of ameloblastoma and 10 cases of adenomatoid odontogenic tumor using the AgNOR method and they found out that the clinical behavior of such lesions is not related to their proliferation index, although the origin of such lesions is different from the origin of leukoplakias.

Madan et al.²⁶ evaluated the cell proliferative activity (AgNOR method) of 30 leukoplakia cases (15 cases with dysplasia and 15 with no dysplasia), 15 cases of squamous cell carcinoma, and 5 cases of normal oral mucosa. The authors found that the NOR count in carcinomas was lower than in dysplastic lesions. In addition, the

mean NOR count was not statistically different between dysplastic and non-dysplastic leukoplakias. Thus, these authors conclude that the average of NORs cannot, as an isolated factor, serve to distinguish normal epithelium from cancerous lesions or oral carcinomas.

Khushbu et al.³² aimed to investigate the rate of cell proliferation and biological aggressiveness of normal epithelium, cancerous lesions (leukoplakias), and malignant lesions (squamous cell carcinomas) using the AgNOR method. However, they found that the amount of NORs does not necessarily indicate tissue malignancy.

Thus, in the present study, the amount of NORs was not significantly related to three established histopathological classifications for oral leukoplakia. Thereby, the AgNOR method should be used with caution when differentiating leukoplakias of different histological grades or when distinguishing between cancerizable oral lesions and malignant lesions.

It is important to note that the present study has limitations regarding the sample, once it consists in only 18 lesions and the fact that the patients were not clinically followed up in order to determine the actual prognosis of the analyzed cases.

CONCLUSION

From the studied sample and applied methodology in the present investigation, it can be inferred that the average number of NORs is not associated with the recognized histopathological classifications for oral leukoplakias.

CLINICAL SIGNIFICANCE

The AgNOR method should be used with caution to determine the clinical treatment of oral leukoplakias, since no agreement was observed between this method and the histopathological classifications available for such lesion.

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