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Área de Estomatologia
FACULDADE DE ODONTOLOGIA DE ARAÇATUBA – UNESP

TAMARA FERNANDES DE CASTRO

$CD4^+$, $CD8^+$ AND $FOXP3^+$ LYMPHOCYTES INFILTRATING ORAL LEUKOPLAKIA: AN IMMUNOHISTOCHEMICAL ANALYSIS

DISSERTAÇÃO DE MESTRADO

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$CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes infiltrating oral leukoplakia: an immunohistochemical analysis

Dissertação apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho", para obtenção do título de Mestre, pelo Programa de Pós-Graduação em Odontologia, área de concentração em Estomatologia.

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"Quando você sonha alto todos os passos parecem ser o primeiro
Então esse é só o primeiro passo mais uma vez
E a gente tem muito pra aprender"

José Tiago Sabino Pereira

Projota

Castro TF. Linfócitos $CD4^+$, $CD8^+$ e $FOXP3^+$ que se infiltram na leucoplasia oral: uma análise imunohistoquimica. [Dissertação]- Araçatuba: UNESP-Universidade Estadual Paulista; 2019.

Resumo

Objetivo: Avaliar a infiltraçãdo dos linfócitos $CD4^+$, $CD8^+$ e $FOXP3^+$ e sua correlação com caracteristicas sociodemográfica, clinicopatologicas e estilo de vida de pacientes com leucoplasias bucais. **Pacientes e métodos:** Oitenta pacientes com diagnóstico de leucoplasia bucal foram incluidos no estudo. Análises retrospectivas foram realizadas para verificar as características sociodemográficos, clinicopatológicos e estilo de vida dos pacientes. O infiltrado linfocitário foi caracterizado por imunoistoquímica com antígenos contra de $CD4^+$, $CD8^+$ e $FOXP3^+$. **Resultados:** Dos 80 pacientes incluidos neste estudo, (60%) eram homens e a idade variou de 25 a 82 anos com idade média de 58,6 anos.Trinta e oito (47.5%) eram idosos, Trinta e dois (40%) eram adultos de meia idade e apenas dez (10%) adultos jovens. Sessenta e um dos pacientes eram fumantes (76.2%) e quarenta e seis eram etilistas (57.5%). Vinte e sete (35.5%) das lesões apresentaram algum grau de displasia epitelial. O grau de displasia epitelial apresentou correlação positiva com a intensidade do consumo do alcool ($p=0.008$). Houve correlação positiva entre os linfócitos $CD4^+$ e $CD8^+$ ($p=0.005$). **Conclusão:** O infiltrado linfocitário não foi relacionado com nenhuma característica clinicopatológica das lecoplasias bucais. Entretanto, o grau de displasia está relacionado ao estilo de vida dos pacientes.

Palavras-chave: Leucoplasia, Linfócito-T, Imunoistoquímica.

Castro TF. $CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes infiltrating oral leukoplakia: an immunohistochemical analysis. [dissertation]- Araçatuba: UNESP- São Paulo State University; 2019.

Abstract

Objective: To evaluate the infiltration of $CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes and their correlation with sociodemographic, clinicopathological and lifestyle characteristics of patients with oral leukoplakia. **Patients and methods:** Eighty patients diagnosed with oral leukoplakia were included in the study. Retrospective analyses were performed in order to verify the sociodemographic, clinicopathologic and lifestyle characteristics. The lymphocytic infiltrate characterization was performed by immunohistochemistry with antibodies against $CD4^+$, $CD8^+$, and $FOXP3^+$ markers. **Results:** Of 80 patients included in the study, 60% were men, and their age ranged from 25 to 82 years, with a mean of 58.6. Thirty-eight patients (47.5%) were elderly, Thirty-two (40%) middle-aged, and only ten (10%) young adults. Sixty-one of the patients were smokers (76.2%) and forty-six were alcoholics (57.5%). Twenty-seven (35.5%) of the lesions presented some degree of dysplasia. The degree of epithelial dysplasia was correlated with the intensity of alcohol consumption ($p=0.008$). A positive correlation was found between $CD4^+$ and $CD8^+$ lymphocytes ($p=0.005$). **Conclusion:** The lymphocytic infiltrate of oral leukoplakia was not correlated with any clinicopathologic characteristic. However, the degree of epithelial dysplasia was correlated with the lifestyle of the patients.

Keywords: Leukoplakia; T-Lymphocytes; Immunohistochemistry

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ED, Epithelial dysplasia

FOXP3, Forkhead Transcription Factor

LI, Lymphocytic Infiltrate

OPML, Oral potentially malignant lesions

OL, Oral leukoplakia

OSCC, Oral squamous cell carcinoma

T regs, Regulatory T cells

UNESP, Universidade Estadual Paulista Júlio de Mesquita Filho

SUMMARY

$CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes infiltrating oral leukoplakia: an immunohistochemical analysis

Lymphocytic infiltrates in oral leukoplakia.

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Introduction

Oral cancer is a global public health problem, responsible for 177,384 deaths worldwide in 2018, corresponding to 1.9 deaths per 100,000 inhabitants (Bray et al., 2018). Oral squamous cell carcinoma (OSCC) is the most common malignant disease of the head and neck, accounting for >90% of all malignancies in the mouth. It is one of the most common tumors worldwide, with approximately 300,000 incident cases each year (Ferlay et al., 2015; Stasikowska-Kanicka, Wągrowska-Danilewicz, Danilewicz, 2018). In 2022, 11,180 new cases of oral cavity cancer in men and 4,010 new cases in women were estimated in Brazil (Inca, 2020). Despite therapeutic advances, 5-year survival remains below 50% in the last 50 years, the main reason being the late diagnosis (Mehrotra, Gupta, Singh, Ibrahim, 2006; Ganly, Patel, Shah, 2012; Singla, Singla, Zaheer, Rawat, Mandal, 2018). Thus, the early diagnosis of oral cancer plays a key role within the disease progression, treatment outcome, patient's quality of life and survival (Mehrotra et al., 2006; Ganly et al., 2012; Liu et al., 2013).

OSCC may develop from oral potentially malignant lesions (OPML), which are lesions with high risk of malignant evolution compared to other benign lesions and the normal oral mucosa (Mehrotra et al., 2006; Ganly et al., 2012; Liu et al., 2013), among which oral leukoplakia (OL) is the most frequent (Liu et al., 2010; Mehrotra et al., 2006; Liu et al., 2013; Bisht, Singh, Sikarwar, Darbari, 2013). According to the World Health Organization (WHO), OL is a white lesion that cannot be clinically or pathologically associated with any other disease (Warnakulasuriya, Johnson, Van der Waal, 2007; Liu et al., 2013; Bisht et al.,

2013). Clinicopathologic characteristics as advanced age, female gender, lesions larger than 2cm, non-homogeneous OL and the presence of epithelial dysplasia (ED), reflect a higher risk to malignant progression (Warnakulasuriya, Ariyawardana, 2016; Speight, Khurram, Kujan, 2018). Moreover, the severity of OL is proportional to the degree of ED (Stasikowska-Kanicka et al., 2018; Lodi, Sardella, Bez, Demarosi, Carrassi, 2006). From all the ED that OL may present, about 3-17% turn into OSCC (Liu et al., 2013; Tilakaratne, Sherriff, Morgan, Odell, 2011). The histological exam is mandatory for the diagnosis of OL and to assess the risk of malignant transformation of OL, which is based on the degree of ED observed (Lodi et al., 2006; Warnakulasuriya et al., 2007; Van Der Waal, 2014; Warnakulasuriya et al., 2016).

Recent studies show that the amount and type of lymphocytic infiltrate (LI) are related to the progression and survival of OSCC patients (Yagyuu et al.,2017; Stasikowska-Kanicka et al., 2018; Kouketsu et al., 2019).

LI is related to the antitumor response and changes in LI quantity and type are associated with the prognosis and survival of patients with colorectal, ovarian and breast cancer (Zhang et al., 2003). The higher amounts of $CD8^+$ cytotoxic T-cells infiltrating tumors has been associated with a better prognosis in several cancers (Chen et al., 2019), as it may be associated with a protective microenvironment (Chaves et al., 2019), whereas LI regulating T cells (Tregs) may promote tumor progression and high Tregs levels are linked to immunosuppression and poor prognosis (Liang at al., 2011; Sun et al., 2016 Stasikowska-Kanicka et al., 2018; Singla et al., 2018). This occurs, at least in part, because Treg cells plays a crucial role in suppressing the anti-tumor immune system (Sakaguchi, 2010; Oleinika, 2013; Johnson et al., 2014), in which

Forkhead Transcription Factor (FOXP3) is a transcription factor and serves as the most specific marker for Treg $CD4^+CD25^+FOXP3^+$ in human cancers and is involved in the development and function of Tregs cells (Sakaguchi, Miyara, Costantino, Hafler, 2010; Liang et al., 2011; Oleinika, Nibbs, Graham, Fraser, 2013; Sun et al., 2016; Stasikowska-Kanicka et al., 2017).

To understand the immunopathogenesis of OPML is essential for cancer research. This study aimed to assess the $CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes infiltrating OL by the immunohistochemistry method and evaluate their correlation with sociodemographic, clinicopathological and lifestyle characteristics of the patients.

Patients and Methods

Patients

This retrospective study included patients diagnosed with OL between 2011 and 2018 at the Oral Oncology Center, São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil. This study was approved by the Research Ethics Committee in human studies (Protocol nº. 03876012.6.0000.5420). The inclusion criteria were patients with clinically and microscopically confirmed diagnosis of primary OL, and who agreed to participate in the study. The exclusion criteria were patients who received previous treatment for OL, or did not accept to participate in the study, and those diagnosed with non-homogeneous leukoplakia. A total of 80 patients were included. The corresponding

sociodemographic, clinicopathologic and lifestyle information was retrieved from the patients' medical records.

Clinicopathologic data collection

Sociodemographic, clinicopathologic, and lifestyle data of the patients were obtained from their individual records. The sociodemographic variables included age, sex, and race. The clinicopathologic variables included location, present or absent epithelial dysplasia (ED) and ED degree of the lesion, which was considered according to the grading proposed by the WHO (Warnakulasuriya et al., 2008) performed by a single pathologist for all cases involved in this study. Lifestyle factors included the status and exposure intensity to tobacco and alcohol. For analysis, the status of tobacco smoking and alcohol drinking were classified as current smoker/drinker, never smoker/drinker; and the intensity of these habits was classified as light, moderate and severe. The average number of cigarette equivalents smoked per day was categorically defined as light (≤19), moderate (20–39), and severe exposure intensity (≥40). Measurements of alcohol consumption were graded as light (0-2 drinks/day), moderate (3-4 drinks/day), and severe exposure intensity (>4 drinks/day) (Rapoport A, Kowalski, 1989).

Immunohistochemistry

So as to evaluate the presence of $CD4^+$ and $CD8^+$ lymphocytes in the OL, immunohistochemical staining was performed on 4µm tissue sections. The sections were deparaffinized and rehydrated. Epitope retrieval was performed by incubation in 10mM citrate buffer (pH= 6.0) for 20min at 55ºC using a steamer. Incubations of 20 minutes with 3% hydrogen peroxide were carried out in order to block endogenous peroxidase activity. The primary antibodies anti-CD4 (clone EPR6855; Abcam, UK; 1:1500 dilution) and anti-CD8 (clone EP1150Y; Abcam, UK; 1:1500 dilution) were incubated for 3 hours. The sections were then incubated with the secondary antibody (Histofine MULTI; Nichirei Biosciences INC., JP) for 30 minutes. Immunostaining was performed immersing the sections in 3,3'-diaminobenzidine (DAB substrate kit; DAKO, USA) and subsequently were counterstained with Harris's hematoxylin, dehydrated, cleared and mounted.

In order to evaluate the presence of $FOXP3^+$ lymphocytes, immunohistochemical staining was performed on 4µm tissue sections. They were then treated with 3% hydrogen peroxide in methanol for 30min at room temperature so as to block endogenous peroxidases. The sections were submitted to an antigenic recovery with 3 heating treatments (100ºC) of 4 min, with intervals of 1 min, with sodium citrate buffer (pH 6.0) in a microwave oven (760W power). In order to block non-specific binding proteins, the sections were incubated in PBS (phosphate-buffered saline) containing 2% bovine serum albumin, for 30 min, at room temperature. The sections were then incubated with primary antibodies for $FOXP3^+$ (clone 236A/E7; Abcam, UK; dilution 1:500), at 4ºC, overnight. The sections were then incubated with the secondary antibody (Histofine MULTI; Nichirei Biosciences INC., JP) for 30 minutes. Immunostaining was performed

immersing the sections in 3,3'-diaminobenzidine (DAB substrate kit; DAKO, USA), which were subsequently counterstained with Harris's hematoxylin, dehydrated, cleared and mounted. Similarly produced sections of the tonsil were used as a positive control for all antibodies. The positive controls for the immunohistochemical staining were prepared using the same specific antibody concentration. The negative controls were produced by replacing the primary antibodies with the antibody diluent.

All samples inserted in parafin used for diagnostic purposes were deposited in the Oral Medicine Biobank, São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil.

Morphometry

One examiner expert on microscopic observation and blinded to the ED information's and the clinical history of the patients assessed the immunohistochemical staining results. T cells were counted manually by means of the ImageJ Java 1.52a software (National Institutes of Health, Rockville, MD; http://imagej.net/ImageJ) in 7-10 high-magnification fields (400x), starting from the center of the histopathological section to 5 right fields and 5 left fields. Subepithelial $CD4^+$, $CD8^+$, and $FOXP3^+$ cells were counted in the lamina propria between the interpapillary ridges and subepithelial areas. The average count was calculated and graded as 1+, 2+, or 3+, i.e., ≤5, 6-19, or ≥20 T cells for each lesion, respectively. (Zhang et al., 2003; Yagyuu et al., 2017).

Statistical analysis

Statistical analysis was performed using GraphPad Prism® version 8.0 (GraphPad Software Inc, La Jolla, CA). Frequency distributions were provided for the clinicopathologic variables. For the correlation of clinical and pathological characteristics and $CD4^+$, $CD8^+$, and $FOXP3^+$ scores, Spearman's rank correlation coefficient test was used. P-values < 0.05 were considered statistically significant.

Results

Patients' characteristics

The characteristics of the patients included in this study are summarized in Table 1. The study included 80 patients, 48 (60%) were men and 32 (40%) were women. The mean age was 58.6 years at initial diagnosis. Thirty-eight (47.5%) individuals were elderly (>60-years old), thirty-two (40%) middle-aged (45-59-years old) and ten (12.5%) young-adults (<45 years old). Fifty-seven patients were white (71.3%), while twenty-three (28.7%) were non-white. The OL most common involved site was buccal mucosa (n = 21; 26.2%).

Forty-nine (64.5%) patients did not exhibit ED, twenty-seven (35.5%) patients showed ED and from four patients these could not be obtained. Low, moderate, and severe dysplasia were identified in sixteen (59.3%), nine (33.3%), and two (7.4%) cases, respectively.

Regarding lifestyle, most patients were current smokers, amounting to sixty-one (76.3%), and nineteen (23.7%) nonsmokers. Thirty patients were considered light smokers (50.8%), eighteen (30.6%) moderate smokers, and only eleven (18.6%) heavy smokers. Forty-six (57.5%) patients with OL were current alcohol drinkers and thirty-four (42.5%) never drinkers. In respect to alcohol consumption, twenty patients were regarded as light drinkers (55.5%), thirteen (36.2%) moderate drinkers, and three (8.3%) heavy drinkers.

Seventy patients (87.4%) showed score 2 and 3 and only ten patients (12.5%) presented score 1 for $CD4^+$ immunoreactivity. Fourteen (31.2%), twenty-seven (60%) and four patients (8.8%) exhibited 1, 2 and 3 scores, respectively, for $CD8^+$ immunoreactivity. Thirty-two (71.2%), eleven (24.4%) and two patients (4.4%) displayed 1, 2 and 3 scores, respectively, for $FOXP3^+$ immunoreactivity.

Table 1. Sociodemographic, clinicopathologic and lifestyle characteristics, and CD4+, CD8+ and FOXP3+ score results of patients with oral leukoplakia.

Variable	N	%
Sex		
Male	48	60.0%
Female	32	40.0%
Age		
Young	10	12.5%
Middle age	32	40.0%
Elderly	38	47.5%
Race		
White	57	71.3%
Non-white	23	28.7%
Location		
Tongue	17	21.2%
Gingiva	12	15.0%
Buccal mucosa	21	26.2%
Palate	12	15.0%
Lip	11	13.8%
Floor of mouth	7	8.8%
ED		
Present	27	35.5%
Absent	49	64.5%
No date	4	-
Degree of ED		
Low	16	59.3%
Moderate	9	33.3%
Severe	2	7.4%
Tobacco smoking status		
Smoker	61	76.3%
Nonsmoker	19	23.7%
Tobacco smoking intensity		
Light	30	50.8%
Moderate	18	30.6%
Heavy	11	18.6%
No date	2	-
Alcohol drinking status		
Drinker	46	57.5%
Nondrinker	34	42.5%
Alcohol drinking intensity		
Light	20	55.5%
Moderate	13	36.2%
Heavy	3	8.3%
No date	10	-
Score CD4		
1	10	12.5%
2	35	43.7%
3	35	43.7%
Score CD8		
1	14	31.2%
2	27	60.0%
3	4	8.8%
Score FOXP3		
1	32	71.2%
2	11	24.4%
3	2	4.4%
TOTAL	80	100%

ED: Epithelial dysplasia;

The correlative study revealed statistically significant correlations between the $CD4^+$ and $CD8^+$ scores of infiltrating cells. What is more, it was found a significant correlation between ED and alcohol drinking intensity (Table 2).Regarding dysplasia, tobacco smoking intensity, tobacco smoking status, and alcohol drinking status, no correlation was found between clinicopathologic and lifestyle characteristics and $CD4^+$, $CD8^+$ and $FOXP3^+$ scores (Table 2).

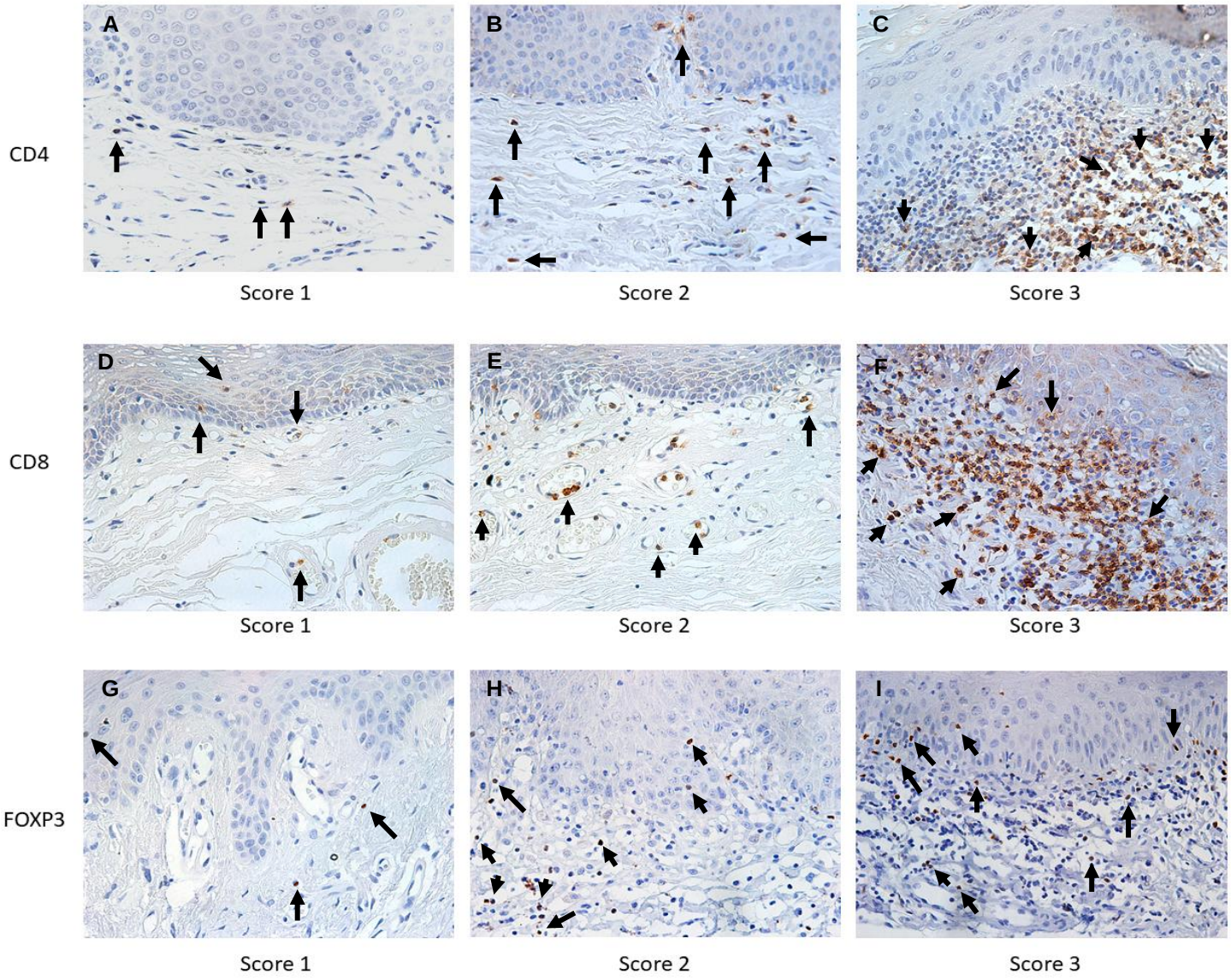
Table 2. The correlation results between clinicopathologic and lifestyle characteristics and $CD4^+$, $CD8^+$ and $FOXP3^+$ scores of patients with oral leukoplakia.

Correlation between	R	p-value	CI
ED vs Tobacco smoking intensity	-0.129	0.330	-0.379 to 0.139
ED vs Tobacco smoking status	0.026	0.821	-0.201 to 0.250
ED vs Alcohol drinking intensity	0.035	0.838	-0.305 to 0.368
ED vs Alcohol drinking status	0.079	0.487	-0.149 to 0.299
ED vs CD4 score	-0.050	0.658	-0.273 to 0.177
ED vs CD8 score	-0.187	0.096	-0.396 to 0.040
ED vs FOXP3 score	-0.035	0.760	-0.258 to 0.192
Degree of ED vs Tobacco smoking intensity	0.017	0.945	-0.440 to 0.466
Degree of ED vs Tobacco smoking status	-0.033	0.871	-0.417 to 0.361
Degree of ED vs Alcohol drinking intensity	0.847	0.008*	0.516 to 0.957
Degree of ED vs Alcohol drinking status	-0.136	0.499	-0.499 to 0.268
Degree of ED dysplasia vs CD4 score	0.015	0.942	-0.377 to 0.402
Degree of ED vs CD8 score	-0.159	0.429	-0.516 to 0.246
Degree of ED vs FOXP3 score	-0.026	0.898	-0.411 to 0.367
Tobacco smoking intensity vs Tobacco smoking status	-0.122	0.356	-0.373 to 0.145
Tobacco smoking intensity vs Alcohol drinking intensity	0.136	0.472	-0.245 to 0.482
Tobacco smoking intensity vs Alcohol drinking status	-0.205	0.119	-0.444 to 0.06
Tobacco smoking intensity vs CD4 score	-0.067	0.614	-0.324 to 0.199
Tobacco smoking intensity vs CD8 score	0.082	0.537	-0.185 to 0.338
Tobacco smoking intensity vs FOXP3 score	0.199	0.131	-0.067 to 0.439
Tobacco smoking status vs alcoholism intensity	-0.149	0.385	-0.463 to 0.198
Tobacco smoking status vs Alcohol drinking status	0.411	1.45e-004	-0.204 to 0583
Tobacco smoking status vs CD4 score	-0.035	0.759	-0.258 to 0.196
Tobacco smoking status vs CD8 score	0.008	0.947	-0.218 to 0.233
Tobacco smoking status vs FOXP3 score	-0.010	0.931	-0.235 to 0.216
Alcohol drinking intensity vs Alcohol drinking status	-0.082	0.634	-0.408 to 0.262
Alcohol drinking intensity vs CD4 score	-0.140	0.416	-0.455 to 0.207
Alcohol drinking intensity vs CD8 score	-0.104	0.548	-0.426 to 0.242
Alcohol drinking intensity vs FOXP3 score	0.067	0.698	-0.276 to 0.395
Alcohol drinking status vs CD4 score	-0.057	0.615	-0.279 to 0.171
Alcohol drinking status vs CD8 score	-0.005	0.964	-0.203 to 0.211
Alcohol drinking status vs FOXP3 score	-0.145	0.200	-0.359 to 0.083
CD4 score vs CD8 score	0.310	0.005*	0.090 to 0.500
CD4 score vs FOXP3 score	0.405	1904e-004	0.197 to 0.578
CD8 score vs FOXP3 score	0.199	0.076	-0.027 to 0.407

CI: confidence interval; ED: Epithelial dysplasia; r: correlation; *p<0.05

$FOXP3^+$ was shown to be nuclear immunoreactive. $CD4^+$ and $CD8^+$ were clearly stained in the cell membrane of the infiltrating cells (Figure 1).

Figure 1. The scores of $CD4^+$, $CD8^+$, and $FOXP3^+$ in OL were detected by immunohistochemistry (400×). (A-C) The CD4+ scores. (D-F) The CD8+ scores. (G-I) The FOXP3+ scores. Arrows: indication of CD4/CD8/FOXP3 immunostaining.



Discussion

Immunological factors have been associated with the development and progression of malignant and premalignant lesions. Therefore, it is imperative to investigate OPML antigenic repertoire, immunological environment, and immune modulation, and identify the regulators of its progression and regression, in order to provide information on the early stages of oncogenesis. Thus, understanding the immunopathogenesis of OL may provide new paths to OSCC immunotherapeutic strategies. In this retrospective study, we analyzed the sociodemographic, clinical-pathological and lifestyle data of patients with OL, and T-lymphocyte types that infiltrated the OL.

OL is the most common OPML and its risk of cancer progression ranges from 0.13 to 34% (Warnakulasuriya et al., 2016), and may depend on clinicopathologic characteristics as sex, age, location and etiology (Warnakulasuriya et al., 2016). According to Warnakulasuriya (2016), older female patients who have OL are more prone to develop malignancies than younger men. In our study, however, patients were older (47.5%) and men (60%). Moreover, the border of the tongue is known to have a higher risk for malignancy, especially from a pre-existing OL (Warnakulasuriya et al., 2016), which was not observed in our sample, since the most prevalent site of OL was the buccal mucosa (26.2%).

Not only is chronic tobacco smoking the main risk factor for OSCC but also for OL occurrence (Wu, Wang, Zhou, 2019). However, a higher risk for malignant progression of OL is attributed to non-smoker patients (Warnakulasuriya et al., 2007). In the present study, most patients were smokers

(76.2%) and drinkers (57.5%). These data, along with the location of most of the OLs included in this study may explain the low rate of ED observed in our study. However, it is important to notice that the ED degree increased with the severity of alcohol consumption.

A more intense LI was observed in OPML compared to OSCC (Johnson et al., 2014), suggesting that in the earlier stages of carcinogenesis, the cellular immune response is recruited to suppress the tumor progression. A hypothesis, still to be investigated, is that the successful malignant progression depends, at least in part, on the tumor cells in order to acquire the ability to escape the immunologic response. The CD4+ T-cell is known to be responsible for activating the cytotoxic T-cell $CD8^+$ in the tumor microenvironment, reflecting a better outcome for patients with more intense infiltrates composed of these cells (Sato et al., 2005; Liang et al., 2011; Johnson et al., 2014; Stasikowska-Kanicka et al., 2018; Singla et al., 2018). On the other hand, the $CD4^+$ $FoxP3^+$ T-cells regulate the immune response by inactivating the $CD8^+$ cytotoxic T-cells (Sun et al., 2016). Numerous studies suggest an association of high intratumoral CD8+ T-cell infiltration with a better clinical outcome, which may be related to a protective microenvironment in OPML and OSCC, corroborating the concept that reduced adaptive immunity facilitates malignant transformations and cancer metastases (Stasikowska-Kanicka et al., 2018; Chaves et al., 2019).

Stasikowska-Kanick et al., (2018) analyzed the amount of $CD4^+$ and $CD8^+$ lymphocytes in patients with OSCC metastasis (m+), OSCC without metastasis (m-), and OL with moderate to severe ED. A significant increase of intratumoral CD4+ cells in OSCC (m+) patients compared with patients with OSCC (m-) and OL was observed. On the other hand, an increase of the $CD8^+$ LI was observed

in OL patients and OSCC patients (m-) in comparison with OSCC patients (m+). In the present study, we did not observe any correlation of the $CD8^+$ LI with the degree of ED. However, the low rate of ED of our sample may have influenced this observation.

Furthermore, Chen et al. (2019) analyzed the amount of $CD8^+$ cells in patients with OSCC and patients with OL. OSCC patients with infiltrating $CD8^+$ cells have been shown to present better survival. Chaves et al. (2019) analyzed $CD4^+$ and $CD8^+$ cells in patients with OSCC, patients with OPML that transformed into OSCC, and patients with OPML that did not develop into OSCC. No significant differences in the $CD4^+$ infiltrate among all groups was observed. However, reduced levels of $CD8^+$ cells in OPML patients were found compared to OSCC patients and a significant increase in $CD8^+$ cells was demonstrated in OPML that progressed to OSCC compared to OPML that did not progress into OSCC. In both studies, the $CD8^+$ LI intensity was proportional to the degree of ED. Thus, the $CD8^+$ IL present in the early OPML stages was considered low or nonexistent. Accordingly, no significant difference for $CD8^+$ score was observed in our study, possibly due to the prevalent low degree of ED.

Sun et al. (2016) analyzed $CD4^+$ lymphocyte status and the association with Foxp3 in OSCC, OL, and oral lichen planus patients. A gradual increase was shown in infiltrating $CD4^+$ and $FoxP3^+$ T-cells in parallel with the progression of ED in OL samples, which suggests that immune suppressor cells influence malignant tumor progression. Notwithstanding, Sun et al., (2016) did not present the location prevalence of OL, or the OL patients' habits, which may have influenced the results. In the present study, neither the $CD4^+$ nor the $FOXP3^+$ T

cells infiltrate were correlated with the degree of ED or any other clinicopathologic characteristic.

In our study, a correlation between $CD4^+$ and $CD8^+$ T cells was shown, which was expected from an adaptive immunological response (Johnson et al., 2014). However, no correlation between $CD4^+$ T-cells and $FoxP3^+$ was found. It is possible to assume that the number of altered cells was not sufficient to exacerbate the $CD4^+$ immune response to such an extent to activate the $FOXP3^+$ pathway, or the intensity of the $CD4^+$ immune response was not sufficient to require $FOXP3^+$ activation. This may have occurred due to the patients' profile in our sample, who did not show risk factors for malignant transformation. Therefore, long-term follow-up of these patients with OL, or the lack of a control group, either OSCC or healthy patients, are considered limitations of the present study. In conclusion, the lymphocyte inflammatory infiltrate, $CD4^+$, $CD8^+$, and $FOXP3^+$, did not correlate with the available clinicopathological data; nonetheless, the intensity of alcohol consumption was correlated with the ED degree. Further investigations must be conducted to better understand the role of the LI on OL and its risk for malignancy. For that, the inclusion of a higher number of lesions with ED must be prioritized, long term follow-up data must be considered, and control groups of OSCC and healthy patients must be included. Moreover, epithelial antigens must be characterized in all the degrees of ED.

Conflict of interest statement

The authors declare that there is no potential conflict of interest regarding this work.

Founding

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Anexo A

Parecer consubstanciado do CEP – FOA/UNESP

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"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

COMITÊ DE ÉTICA EM PESQUISA

CERTIFICADO

Certificamos que o Projeto ***"Detecção do HPV por nPCR em leucoplasias bucais: Estudo caso-controle"***, sob a responsabilidade do Pesquisador **GLAUCO ISSAMU MIYAHARA**, está de acordo com os Princípios Éticos em Pesquisa e foi aprovado em 27/05/2011, de acordo com o Processo FOA-01034/2011.

Araçatuba, 06 de junho de 2011.

ALESSANDRA MARCONDES ARANEGA
Vice-Coordenadora do CEP

AMA/wbm.

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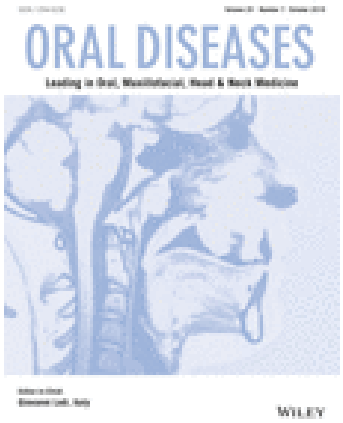
Anexo – B

Periódico de interesse para submissão

Oral Diseases



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