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AVALIAÇÃO DA EXPRESSÃO DO KI-67 E BMI-1 EMLEUCOPLASIAS BUCAIS DISPLÁSICAS E NÃO DISPLÁSICAS

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AVALIAÇÃO DA EXPRESSÃO DO KI-67 E DO BMI-1 EM LEUCOPLASIAS BUCAIS DISPLÁSICAS E NÃO DISPLÁSICAS

Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Rio Grande do Sul, como requisito à obtenção do título de mestre em Odontologia.

Orientador: Prof. Dr. Vinicius Coelho Carrard

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RESUMO

KLEIN, Isadora Peres. **Avaliação da expressão do Ki-67 e do BMI-1 em leucoplasias bucais displásicas e não displásicas.** 2015. 43f. Dissertação (Mestrado) - Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2015.

Leucoplasia bucal (LB) é uma desordem potencialmente maligna, com risco de transformação maligna que varia de 0,13% a 17,5%. Muitos estudos vêm buscando estabelecer biomarcadores capazes de predizer o potencial de transformação maligna dessa lesão. O Ki-67 é uma proteína não-histônica nuclear que tem sido amplamente utilizada para avaliar proliferação celular. O BMI-1 é uma proteína considerada um marcador essencial para a manutenção das propriedades de autorrenovação e da tumorigenicidade do carcinoma espinocelular. O objetivo principal desse estudo observacional transversal foi avaliar proliferação e imortalização celulares em LB a partir da marcação imunoistoquímica do Ki-67 e do BMI-1, comparando lesões displásicas com não displásicas. Casos de LB não displásica - LBND (n=28), LB displásica - LBD (n=33) foram selecionados a partir de prontuários de pacientes e comparados com mucosa clinicamente normal - MN (n=9), hiperplasia inflamatória - HI (n=17) e carcinoma espinocelular - CEC (n=19). Os diagnósticos histopatológicos foram confirmados a partir da revisão de cortes histológicos corados por hematoxilina e eosina. Adicionalmente, cortes histológicos foram submetidos à técnica imunoistoquímica para avaliação de Ki-67 e BMI-1. Para a quantificação foi considerado o percentual de células positivas por 1000 células para o CEC e 1500 células para os demais grupos. O percentual de imunomarcação de Ki-67 e de BMI-1, quando avaliadas todas as camadas epiteliais em conjunto, foi mais alto no CEC quando comparado aos demais grupos (Kruskal-Wallis, $p<0.05$). A expressão de Ki-67 foi maior em LBND, LBD e HI quando comparada com MN (Kruskal-Wallis, $p<0.05$). Além disso, a imunomarcação de BMI-1 foi maior em LBD quando comparada com MN, quando analisadas todas as camadas em conjunto (Kruskal-Wallis, $p<0.05$). A partir da avaliação das camadas epiteliais separadamente, observou-se aumento da expressão de BMI-1 na camada parabasal e suprabasal em LBND quando comparada com MN. Houve correlação positiva entre Ki-67 e BMI-1 (Correlação de Spearman, $R=0.37$, $p<0.05$). Conclui-se que a proliferação e as alterações na transição epitélio-mesênquima são eventos relacionados e que estão presentes desde estágios precoces e se acentuam nos estágios mais tardios da carcinogênese.

Palavras-chave: Leucoplasia bucal. Evolução clínica. Carcinoma espinocelular**.**

ABSTRACT

KLEIN, Isadora Peres. **Evaluation of Ki-67 and BMI-1 expression in non-dysplastic and dysplastic oral leukoplakias.** 2015. 43 pages. Dissertation (Master`s Degree) – Dental School, Federal University of Rio Grande do Sul, Porto Alegre, 2015.

Oral leukoplakia (OL) is potentially malignant disorder, with a risk of malignant transformation that ranges from 0.13% to 17.5%. Many biological markers have been used as an attempt to predict malignant transformation, but no reliable markers have been established so far. The Ki-67 is a nuclear non-histone regarded as reliable marker of proliferating cells .BMI-1 is a protein considered as an essential marker for maintenance of properties self-renewability and tumorigenicity of squamous cell carcinoma. The main aim this cross-sectional observational study was to evaluate cell proliferation and immortalization in oral leukoplakia, comparing non-dysplastic and dysplastic lesions. Cases of non-dysplastic – Non-dys OL (n=28), dysplastic – Dys OL (n=33) records were selected and compared with normal oral mucosa – NOM (n=9), inflammatory hyperplasia –IH (n=17), and oral squamous cell carcinoma - OSCC (n=19). The histopathological diagnosis was confirmed by the revision of Hematoxilin and Eosin stained slides. Additionally, histological sections were submitted to immunohistochemical technique for evaluation of Ki-67 and BMI-1. The labeling index was determined by counting the labeled nuclei of 1000 cells for OSCC cases and 1500 for the others comparison groups. Ki-67 and BMI-1 immunolabeling percentage were higher in OSCC in comparison of others groups when all epithelial layers were evaluated together (Kruskal-Wallis, $p<0.05$). Ki-67 immunolabeling increased in Non-dys OL, Dys OL and IH when compared to NOM (Kruskal-Wallis, $p<0.05$). Furthermore, BMI-1 immunolabeling was higher in Dys OL relation to NOM in the analysis of all epithelial layers together (Kruskal-Wallis, $p<0.05$). When the evaluation considered the epithelial layer separately, an increased BMI1 expression was observed in the parabasal and suprabasal layers of Non-dys OL when compared to NOM. A significant positive correlation was found between Ki-67 and BMI-1 (Spearman correlation coefficient, $R=0.37$, $p<0.01$). In conclusion, the present findings support that proliferation and changes toward epithelial-to-mesenchymal transition increase gradually since early stages of oral carcinogenesis.

Keywords: Oral leukoplakia. Clinical evolution. Oral squamous cell carcinoma.

SUMÁRIO

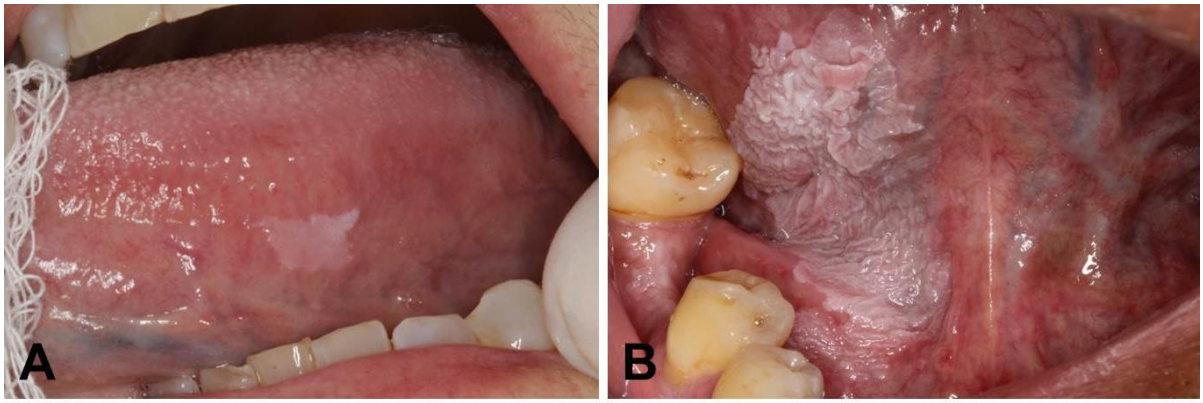
1. ANTECEDENTES E JUSTIFICATIVA

A Leucoplasia bucal é definida como uma placa ou mancha predominantemente branca que não pode ser classificada clinica ou patologicamente como qualquer outra doença (1). Esta lesão é incluída no grupo de desordens potencialmente malignas, visto que apresenta risco de transformação em carcinoma espinocelular (2). A leucoplasia é a lesão mais frequente dentre as desordens potencialmente malignas de boca, com prevalência estimada entre 0,42 e 5% (3-5). Essa lesão é mais frequente em indivíduos do sexo masculino (6, 7) e na faixa etária que vai dos 40 aos 60 anos (8, 9).

O consumo de tabaco é o fator de risco mais importante para o desenvolvimento da leucoplasia bucal (10). Pacientes que fumam mais de 20 cigarros por dia apresentam um risco de desenvolver leucoplasia seis vezes maior do que os não fumantes (6). Com relação à participação de outros fatores na ocorrência dessa lesão, como o consumo de bebidas alcoólicas, não há consenso na literatura (11, 12).

A leucoplasia pode ocorrer em qualquer região da boca, entretanto os sítios anatômicos mais acometidos são a mucosa jugal, o assoalho de boca, o palato e a língua (13, 14). Clinicamente, essas lesões podem se apresentar como manchas ou placas de coloração cinzenta ou branco-acinzentada e terem apresentação única ou múltipla. As lesões são classificadas como: homogêneas (Figura 1.A), quando apresentam superfície lisa, fina e uniforme sem áreas avermelhadas ou não homogêneas (Figura 1.B), que se caracterizam por apresentarem superfície irregular/rugosa (15) ou áreas avermelhadas, sendo chamadas eritroleucoplasia, leucoplasia mosqueada, ou leucoplasia salpicada (16).

Figura 1 - Imagens clínicas de diferentes apresentações clínicas de leucoplasia: A) leucoplasia homogênea localizada na borda de língua; B) leucoplasia não homogênea localizada no assoalho bucal e no ventre de língua.

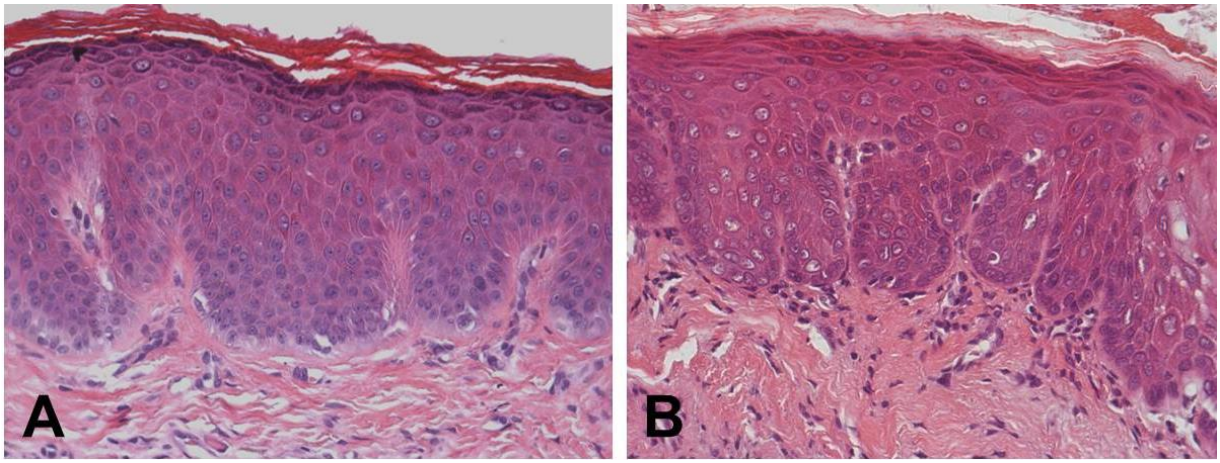


Fonte: Patologia Bucal/UFRGS

Microscopicamente, as leucoplasias podem apresentar diferentes alterações morfológicas no tecido epitelial. Essas alterações resultam de distúrbios do processo de renovação epitelial (ritmo de proliferação e diferenciação celular) (17). Estes distúrbios são classificados de acordo com suas características morfológicas em hiperplasia, hiperceratose, acantose e displasia epitelial. No diagnóstico histopatológico de uma leucoplasia, esses distúrbios de maturação epitelial podem estar presentes isoladamente ou de forma combinada. Quando isso ocorre, deve ser valorizada a alteração mais grave (18, 19).

A hiperplasia epitelial caracteriza-se pelo aumento do número de células do tecido com manutenção do seu padrão morfofuncional. A hiperceratose indica aumento da espessura da camada de ceratina que pode ser do tipo paraceratina ou ortoceratina. A acantose é o espessamento da camada espinhosa do epitélio, resultando no encurtamento das papilas conjuntivas tendendo à planificação da interface epitélio-conjuntivo (19, 20). A displasia epitelial se caracteriza pela presença de alterações arquiteturais e celulares que podem se estender por toda espessura do epitélio (13). A displasia epitelial pode ser classificada, de acordo com a extensão das alterações, como leve, moderada e severa. Considera-se displasia leve a situação em que os distúrbios arquiteturais limitam-se ao terço inferior do epitélio, acompanhados por alterações celulares discretas. Já na displasia moderada as alterações arquiteturais e celulares atingem os dois terços inferiores do epitélio. Na displasia severa os distúrbios arquiteturais ultrapassam os dois terços do epitélio mostrando atipia celular associada (1,19).

Figura 2 - Fotomicrografias ilustrando alterações microscópicas encontradas em leucoplasias: A) hiperplasia epitelial com duplicação da camada basal, hipercromatismo e hiperceratose (leucoplasia não displásica); B) alterações arquiteturais e celulares como hipercromatismo, pleomorfismo celular e nuclear, disceratose, perda da adesão intercelular, papilas epiteliais em forma de gota, alteração da estratificação epitelial e hiperceratose, envolvendo dois terços do compartimento epitelial, caracterizando displasia epitelial moderada (leucoplasia displásica).



Fonte: Patologia Bucal/UFRGS

A taxa de transformação maligna varia 0,13-17,5%, em períodos de observação de 1 a 30 anos (21, 22). Alguns fatores parecem contribuir para o maior risco de malignização, dentre eles: faixa etária acima de 50 anos (23), sexo feminino (2), localização na mucosa da língua ou do assoalho de boca (2, 24), tamanho da lesão maior que 200 mm^2 (2, 15), tipo clínico não homogêneo (2, 4, 15) ou presença de displasia epitelial (2, 25).

A relação entre presença de displasia epitelial e transformação maligna ainda não está clara. Alguns autores sugerem que as leucoplasias displásicas apresentam maior risco de malignização (2, 9), porém outros autores não encontraram essa relação (15, 26). Diversos estudos demonstraram que essa discrepância pode estar relacionada à subjetividade associada com o diagnóstico de displasia epitelial (27-30).

A previsibilidade da evolução de leucoplasias ainda permanece em discussão. Em função disso, o risco malignização justifica a realização de consultas de acompanhamento periódico (2). Atualmente, vem se buscando formas alternativas para monitoramento dos pacientes com leucoplasia, a fim de

se estabelecer biomarcadores capazes de predizer em quais casos haverá transformação maligna.

Dentre os aspectos avaliados nestes estudos, proliferação celular e apoptose são eventos amplamente estudados (31- 34). O Ki-67, uma proteína não-histônica nuclear expressa em todas as fases do ciclo celular, exceto na G0 (35) tem sido amplamente utilizado para avaliar proliferação celular em displasia epitelial e em carcinomas espinocelulares de boca (26, 31, 36, 37). A partir destes estudos têm sido demonstrado aumento da atividade proliferativa em leucoplasia (31, 38, 39). Além disso, observou-se maior expressão do Ki-67 na camada suprabasal do epitélio em casos de displasia epitelial severa (40).

Além do Ki-67, outros biomarcadores como ciclina D1, AgNOR, BrdU, CENP-F, ERK e PCNA têm sido utilizados para avaliar proliferação celular em leucoplasias (32, 37, 41-43). Sabe-se que a atividade proliferativa é maior em leucoplasias quando comparadas com a mucosa normal (17, 26, 31, 32, 37, 41, 43). Além disso, alguns estudos mostraram maior proliferação celular em displasias severas, sugerindo que existe uma relação direta com o processo de carcinogênese (26, 37, 41, 43). Contudo, não há consenso na literatura com relação a esse aspecto (31, 44, 45). Entretanto, não está claro se as leucoplasias com maior proliferação celular têm um comportamento diferenciado (31, 32), e maior risco de transformação maligna ao longo do tempo.

Outro evento importante para manutenção da atividade proliferativa durante a carcinogênese é a imortalização celular. Em um estudo, a inibição da apoptose foi mais presente em leucoplasias que sofreram transformação maligna, sendo sugerida como fator prognóstico (33). Isso sugere que, ao longo da carcinogênese, o desequilíbrio entre proliferação celular e apoptose parece ser uma etapa fundamental (46-48).

Diversos estudos têm mostrado que a proliferação celular aumenta progressivamente ao longo da carcinogênese. Esse descontrole da proliferação celular está intimamente relacionado ao aumento da expressão da telomerase (49, 50). Fisiologicamente, esta enzima atua restaurando porções de DNA perdidas (telômeros) durante a divisão celular, sendo essa capacidade perdida gradualmente com o envelhecimento (51). Ao longo da carcinogênese, a telomerase é reativada inibindo o envelhecimento celular e levando a imortalização. Em vários tipos de

câncer, verifica-se que as células reativam a telomerase, possibilitando a ocorrência de muitas divisões celulares e, mesmo assim, permanecem vivas (49, 52).

Os mecanismos de controle do balanço entre proliferação celular e imortalização são complexos e envolvem diversas macromoléculas. O BMI-1 é uma proteína da família polycomb (PCG) necessária para a manutenção da autorrenovação celular. O papel dos genes PCG e das proteínas associadas aos mesmos na carcinogênese tem sido discutido (53, 54, 55).

O BMI-1 tem sido considerado um marcador relacionado às propriedades de autorrenovação da tumorigenicidade do câncer de cabeça e pescoço (56, 57). Essa proteína tem sido estudada como uma oncoproteína associada com mau prognóstico em vários tipos de câncer. Além disso, tem sido associada com a iniciação e progressão de diversos tipos de tumor, tais como câncer de orofaringe, nasofaringe e de próstata (58-60). Um estudo prévio mostrou um aumento da expressão BMI-1 em lesões pré-malignas de brônquios e em SCC (61). Em relação ao carcinoma espinocelular de boca, observou-se alta expressão de BMI-1 quando comparado à mucosa normal, suportando que a imortalização é um evento importante durante a carcinogênese e um evento necessário a sobrevivência das células cancerosas (62, 63).

Poucos estudos avaliaram o papel da imortalização no curso de leucoplasias e eritroplasias bucais por meio da expressão de BMI-1 (62, 64-66), ALDH1 (64) e ABCG2 (65, 66). Estes estudos mostraram que a maior imunomarcação apresentou associação com transformação maligna em casos de leucoplasia, indicando que estes marcadores tem valor preditivo. Contudo, em eritroplasias somente a co-expressão de BMI-1 com ALDH1 teve associação com malignização. Além disso, Kang et al. (2007) indicou haver maior expressão do BMI-1 em displasias epiteliais de boca em comparação com mucosa clinicamente normal.

Estudos correlacionando as imunomarcações de BMI-1 com Ki-67 em leucoplasias bucais são escassos. Além disso, estes estudos não costumam correlacionar a ocorrência de imortalização com as características clínicas consideradas sugestivas de comportamento mais agressivo como tamanho maior do que 200mm^2, tipo clínico (não homogêneo) e localização (língua e assoalho bucal), bem como características histopatológicas (ausência e presença de displasia epitelial).

2. OBJETIVOS

2.1 Objetivo geral

Avaliar proliferação celular e imortalização celular em leucoplasia bucais a partir da marcação imunoistoquímica de Ki-67 e BMI-1, respectivamente.

2.2 Objetivos específicos

- Avaliar se a presença de displasia epitelial interfere na atividade proliferativa e na ocorrência de imortalização em leucoplasia bucais.

- Comparar a marcação imunoistoquímica de BMI-1 e Ki-67 em leucoplasia bucais com mucosa clinicamente normal, hiperplasia inflamatória e carcinoma espinocelular.

- Avaliar se a marcação imunoistoquímica de BMI-1 e Ki-67 em leucoplasia bucais tem relação com evolução clínica.

- Avaliar se tipo clínico, localização e tamanho das leucoplasia interferem na atividade proliferativa e na ocorrência de imortalização em leucoplasia bucais.

3. ARTIGO CIENTÍFICO

Artigo apresentado de acordo com as normas do periódico Journal of Oral Maxillofacial Surgery (Qualis Odontologia A2 – Ano base 2014, Fator de impacto 1.28).

Title page

Increase of Ki-67 and BMI-1 expression in oral leukoplakias

Keywords: Oral leukoplakia. Clinical evolution. Cell proliferation. Carcinogenesis.

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Abstract

Purpose: The aim of this study was to evaluate cell proliferation and immortalization in oral leukoplakia (OL), comparing non-dysplastic (Non-dys OL) and dysplastic lesions (Dys OL).

Materials and Methods: The investigators implemented a cross-sectional observational study. Paraffin-embedded tissue blocks of 28 Non-dys OL, 33 Dys OL, 9 normal oral mucosas (NOM), 17 inflammatory hyperplasia (IH) and 19 oral squamous cell carcinomas (OSCC) were stained for Ki-67 and BMI-1 using immunohistochemistry. The percentages of positivity for 1000 cells for OSCC and 1500 cells for IH, Non-dys OL, Dys OL have been evaluated. Apart from OSCC group, a complementary analysis assessed basal, parabasal and suprabasal layer cells separately. Statistical analysis was performed using Kruskal-Wallis/Dunn. P value was set at .05.

Results: A gradual increase in BMI-1 and K-i67 expression were found along oral carcinogenesis. The immunolabeling for those markers was higher in OSCC when compared with the other groups, when all epithelial layers were analyzed together (Kruskal-Wallis, $p<0.05$). Ki-67 expression percentage was higher in Non-dys OL and Dys OL and compared to NOM (Kruskal-Wallis/Dunn, $p<0.05$). In contrast, expression of BMI-1 was higher in Dys OL comparison of NOM (Kruskal-Wallis/Dunn, $p<0.05$). BMI-1 immunolabeling increased in parabasal and in suprabasal layers of leucoplakias in relation to normal epithelium (Kruskal-Wallis, $p<0.05$). No differences were observed in expression of both markers when non-dysplastic and dysplastic leucoplakias were compared. There was a significant positive correlation between Ki-67 and BMI-1 (Spearman correlation coefficient, $R=0.37$, $p<0.01$).

Conclusions: The present findings indicate that BMI-1 expression increase in early oral carcinogenesis and appear to be associated to occurrence of dysplastic changes. Furthermore, our findings indicated that both Ki-67 and BMI-1 are directly correlated and play a role in initiation and progression of OSCC.

Introduction

Oral leukoplakia (OL) is a potentially malignant disorder, with a risk of malignant transformation ranging from 0.13% to 17.5% [1-3]. Histopathologically, this lesion is characterized by a variety of epithelial disorders that may include epithelial dysplasia. The presence of epithelial dysplasia has been considered as one of the most important predictive factors for OL prognosis [1, 4, 5]. However, the criteria to define the presence or to grade epithelial dysplasia are subjective and difficult to reproduce [6-9]. Currently, many biological markers are used in an attempt to predict malignant transformation, but no reliable markers have been established so far [10]. Therefore, there is a need for studies in order to improve the knowledge of biological behavior of OL and oral carcinogenesis.

Ki-67 is a nuclear non-histone protein expressed in the G1, S, G2, and M phases of the cell cycle. Ki-67 is considered an accurate marker of cell proliferation, and it reflects the total growth fraction in different tissues [11]. It has been shown that the expression of Ki-67 correlates with severity of epithelial dysplasia and histological grading of oral squamous cell carcinoma (OSCC) [12, 13].

BMI-1 protein belongs to polycomb group family (PcG) that mediates gene silencing by regulating chromatin structure[14-16] .It plays a role in cell cycle regulation, cell immortalization, cell senescence and epithelial mesenchymal transition. [17,18]. BMI-1 has been associated with initiation and progression of various types of tumor, such as oropharyngeal [17], nasopharyngeal [18] and prostate [19] cancer correlating with poor prognosis in cancer patients. Furthermore, BMI-1 expression increase was found in bronchial premalignant precursor lesions and SCC. Hence, it might be suggested that its presence in proliferating neoplastic cells is an early event in lung carcinogenesis [20]. In OSCC cells, this marker is overexpressed when compared with normal oral mucosa cells and it is assumed to influence cell proliferation and survival in oral carcinogenesis [21].

The aim of the present study was to evaluate the presence of cell proliferation and immortalization by comparing non dysplastic (Non-dys OL) with dysplastic (Dys OL). A further aim was to correlate the occurrence of those events with risk factors, histological grade, and prognosis throughout follow-up.

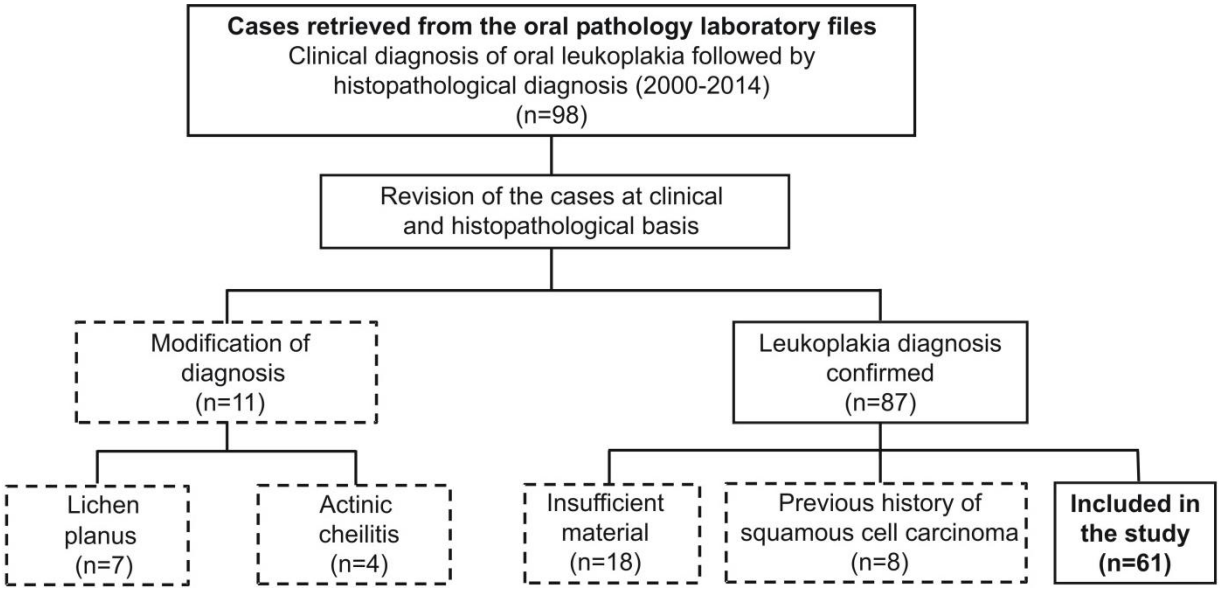
Materials and Methods

Patients and Tissue Specimens

Ninety-eight cases of OL were analyzed in this study. All patients were selected from the archives of the Laboratory of Pathology of the Hospital de Clínicas de Porto Alegre and of the department of Oral Pathology of the School of Dentistry of the Federal University of Rio Grande do Sul, received between January 2000 and December 2014. The study protocol was approved by the Research and Ethics Committee of the Hospital de Clínicas de Porto Alegre, Rio Grande do Sul, Brazil (Human Research Ethics Committee approval no. 140050).

Patient records were evaluated, and information on demographics, risk factors, clinical presentation, treatment, and prognosis throughout the follow-up period were collect. The follow-up period was defined as the time from diagnosis until the last visit to the hospital. Table 1 summarizes the characteristics of these patients. After the application of the eligibility criteria (complete information in the above cited medical records), 61 cases of OL were included in the study. The exclusion criteria of OL are detailed in Figure 1. Nine cases of normal oral mucosa (NOM) obtained during surgical removal of unerupted third molars, 17 cases of inflammatory hyperplasia (IH), and 19 cases of OSCC were used as comparison groups. IH cases have been included as a reference of benign lesion that has cell proliferation increase but no potential for malignant transformation.

Figure 1 - Flowchart of sampling strategy, depicting the criteria to select the study sample. After revision, the cases in which oral leukoplakia was confirmed were submitted to a strict evaluation to define if the amount of tissue was enough to prepare the required number of histological sections. Cases with previous history of squamous cell carcinoma were discarded.



Histopathological analysis

All selected samples had been routinely fixed in 10% neutral formalin, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. Five-µm paraffin sections were cut and mounted on microscope glass slides. All H&E sections were reviewed by two pathologists (V.C.C. and I.P.K.) blinded for the clinical data, the diagnosis was confirmed, and the slides for the quantitative evaluation were selected.

In OL, epithelial dysplasia was diagnosed according to the criteria and definition proposed by Barnes et al (2005): cases in which the disorders were limited to the proliferative zone were considered mild dysplasia; cases in which dysplastic signs were present in up to half of the epithelium thickness were considered moderate dysplasia; and cases in which the atypical proliferative zone encompassed up to three fourths of the epithelium were considered severe dysplasia (Table 1). In some cases, which were graded differently, a consensus was reached. All OSCC cases were revised by the same pathologists, who were unaware of the clinical data.

Immunohistochemistry

Tissue sections from formalin-fixed, paraffin-embedded tissue blocks of NOM, IH, OL, and OSCC were mounted on positively-charged glass slides. Immunohistochemical staining for BMI-1 and Ki-67 proteins was performed using the following antigen retrieval system. The anti-Ki-67 (1:50, MIB-1, DAKO) and anti-BMI-1 monoclonal antibody (1:100 dilution, ab14389; Abcam) was used to detect Ki-67 and BMI-1 expression. A negative control was performed in all cases by omitting the primary antibody, which in all instances resulted in negative immunoreactivity. The positive controls for BMI-1 and Ki-67 were human appendix and reactive lymph node tissue, respectively [23, 24].

Evaluation of immunostainings

Positive nuclear immunostaining was evaluated in the basal layer (single row of cells directly in contact with the basal membrane), parabasal layer (two rows of cells immediately above the basal layer), and suprabasal layer (cell layer above the parabasal layer) by determining the percentage of positively stained cells. The cuboidal and palisading shape was considered as a complementary criterion to define the basal cell layer. Images of the selected fields were captured using a conventional light microscope (CX41RF model, Olympus Latin America, Inc., Miami, Florida, USA) coupled to a camera (QColor 5, Coolet, RTV, Olympus Latin America, Inc., Miami, Florida, USA) and connected to a computer (Dimension 5150, Dell, Porto Alegre, RS, Brazil). The images were analyzed using the QCapture software program (Quantitative Imaging Corporation, Inc., Surrey, DC, 150 Canada), version 2.81. The number and percentage of positive cells were assessed in each case. The quantitative analysis involved analyzing images of the slides using the same imaging system described above. The evaluation was performed under high-power magnification (×400). All marked cells were considered positive when nuclear staining was observed, regardless of staining intensity. The labeling index (LI) was determined by counting the labeled nuclei of 500 basal layer cells, 500 parabasal layer cells, and 500 suprabasal layer cells [11]. For OSCC cases, LI was determined by counting 1000 cells without distinction of layers. The percentage of Ki-67 positivity was calculated based on the number of positive cells in each case.

Statistical Analysis

Since quantitative variables did not follow a Gaussian distribution, Kruskal-Wallis test followed by Dunn's test for multiple comparisons was performed. For comparisons of scores distribution among the groups, chi-square test was used. The linear regression method was used to determine independent predictors of OL. Spearman's correlation analysis was used to determine the relation between BMI-1 and Ki-67 expression when grouping all cases. Statistical calculations were performed using the SPSS Statistics software, Version 18.0. $p<0.05$ was considered to be statistically significant.

Results

Demographic and clinical characteristics

Table 1 shows the demographic and clinical characteristics of the 61 OL and 19 OSCC patients. The cases were grouped as tongue/floor of the mouth and other sites [25]. It was shown that OSCC group has a higher percentage of cases (Student`s t test, p=0.02,) in tongue or floor of the mouth (63.2 %) when compared to OL group (31.6%).

Table 2 shows characteristics of OL without and with OSCC development during the follow-up period (56.9±33.0, ranging from 12 to 156 months). Among them, the information on evolution was no available in 21 cases (34.4%). Four out of 40 OL patients who were followed up (10.0%) developed OSCC. Three of them had lesions located in the tongue or floor of the mouth and three displayed severe epithelial dysplasia at microscopic evaluation. Twenty-two (55.0%) OL patients had good prognosis and 18 (45.0%) poor prognosis. Good prognosis was considered when the patient did not present new lesions and/or OSCC development. Poor prognosis was considered for those patients who presented new lesions and/or underwent OSCC development. The OL patients that developed OSCC presented a higher percentage of severe epithelial dysplasia (Chi-square test, $p<0.01$) when compared to those without OSCC development.

Table 1. Demographic and clinical characteristics of the OL and OSCC patient.

Characteristic	OL	OSCC	p
All patients, no.(%)	61	19	
Age, years			
Mean	58.0	59.4	0.65*
Standard deviation	12.8	10.9	
Range (minimum-maximum)	26-81	39-82	
Gender, no(%)			
Female	26 (42.6)	6 (31.6%)	0.56**
Male	35 (57.4)	13 (68.4%)	
Tobacco habits, no(%)			
Never	8 (16.3)	2 (13.3%)	0.71**
Past and present	41 (83.7)	13 (86.7%)	
Unknown	12	4	
Alcohol consumption, no(%)			
Never	8 (17.4)	3 (25.0%)	0.68**
Past and present	38 (82.6)	9 (75.0%)	
Unknown	15	7	
Location, no(%)			
Tongue / floor of the mouth	19 (31.6)	12 (63.2%)	0.02**
Other locations	41 (68.4)	7 (36.8%)	
Unknown	1	0	
Clinical type, no(%)			
Homogeneous	32 (60.4)	-	
Non homogeneous	21 (39.6)	-	-
Unknown	8	-	
Lesion size, no(%)			
<2 cm	30 (60.0)	-	
≥2 cm	20 (40.0)	-	-
Unknown	11	-	
TNM, no(%)			
I/II	-	5(27.8%)	-
III/IV	-	13(72.2%)	
Unknown	-	1	
Presence of epithelial dysplasia, no(%)			
No	28 (45.9)	-	-
Yes	33 (54.1)	-	
Mild	18(29.5)	-	
Moderate	8(13.1)	-	
Severe	7(11.5)	-	

*Student's t test; **Chi-square test.

Table 2. Demographic and clinical characteristics of the OL with and without OSCC development

	OL patients without OSCC development	OL patients with OSCC development	P
Age			
Mean (SD)	56.3 (13.0)	52.7 (15.9)	0.61*
Min-Max	26-79	31-68	
Gender			
Male	23 (63.9)	2 (50.0)	0.62**
Female	13 (36.1)	2 (50.0)	
Location			
Tongue/floor of the mouth	11 (30.6)	3 (75.0)	0.11*
Others	25 (69.4)	1 (25.0)	
Clinical type			
Homogeneous	20 (64.5)	1 (33.3)	0.54**
Non-homogeneous	11 (35.5)	2 (66.7)	
Unknown	5	1	
Lesion size			
<2cm	17 (54.8)	1 (50.0)	1.00**
≥2cm	14 (45.2)	1 (50.0)	
Unknown	5	2	
Presence of epithelial dysplasia			
No	17 (47.2)	1 (25.0)	0.01**
Yes	19 (52.8)	3 (75.0)	
Mild	12 (33.3)	0	
Moderate	5 (13.9)	0	
Severe[a]	2 (5.6)	3 (75.0)	

*Student's t test; **Chi-square test.
[a] A higher percentage of severe dysplasia was found in OL patients who developed OSCC**.**

Immunohistochemical analysis

Ki-67 is higher in IH, OL and OSCC in relation to NOM

The percentage of positive cells was gradually higher from NOM (6.3%) to IH (21.8%), OL (23.4%), and OSCC (64.8%) when the analysis considered cells of all layers. Dys OL cases displayed a higher Ki-67 immunolabeling (28.2%) when compared with Non-dys OL cases (21.7%), but that difference was not statistically significant.). Figure 2 shows representative images of Ki-67 immunolabeling. Moreover, the medians were statistically higher (Kruskal Wallis, $p<0.01$) in OSCC when compared with NOM, IH, and Non-dys and Dys OL, and in Non-dys and Dys OL when compared with NOM (Figure 3.A). The basal, parabasal, and suprabasal layers were also analyzed separately and compared in NOM, IH, and Non-dys and Dys OL. These findings are shown in Table 3.

BMI-1 increases in OL and OSCC

As shown for Ki-67 immunolabeling, an increasing positivity was observed from NOM (49.7%) to IH (61.8%), OL (71.8%), and OSCC (84.8%). Non-dys OL cases presented lower BMI-1 immunolabeling (69.2%) when compared with Dys OL cases (73.4%), but that difference was not statistically significant. However, the medians were statistically higher (Kruskal Wallis, $p<0.01$) in Dys OL when compared with NOM, and in OSCC when compared with NOM, IH, and Non-dys and Dys OL (Figure 3.B). Similar findings were reported when the parabasal and suprabasal layers were considered separately. The comparisons based on the basal layer cells showed no differences among the groups (Table 4). Representational figures of BMI-1 immunolabeling are shown in Figure 2.

Figure 2. Gradual increase of Ki-67 and BMI-1 expression is observed from to normal oral mucosa (NOM) to oral squamous cell carcinoma (OSCC). Representative photomicrographs of NOM, inflammatory hyperplasia (IH), non-dysplastic oral leukoplakia (Non-dys OL), dysplastic oral leukoplakia (Dys OL) and OSCC. Original magnification ×400.

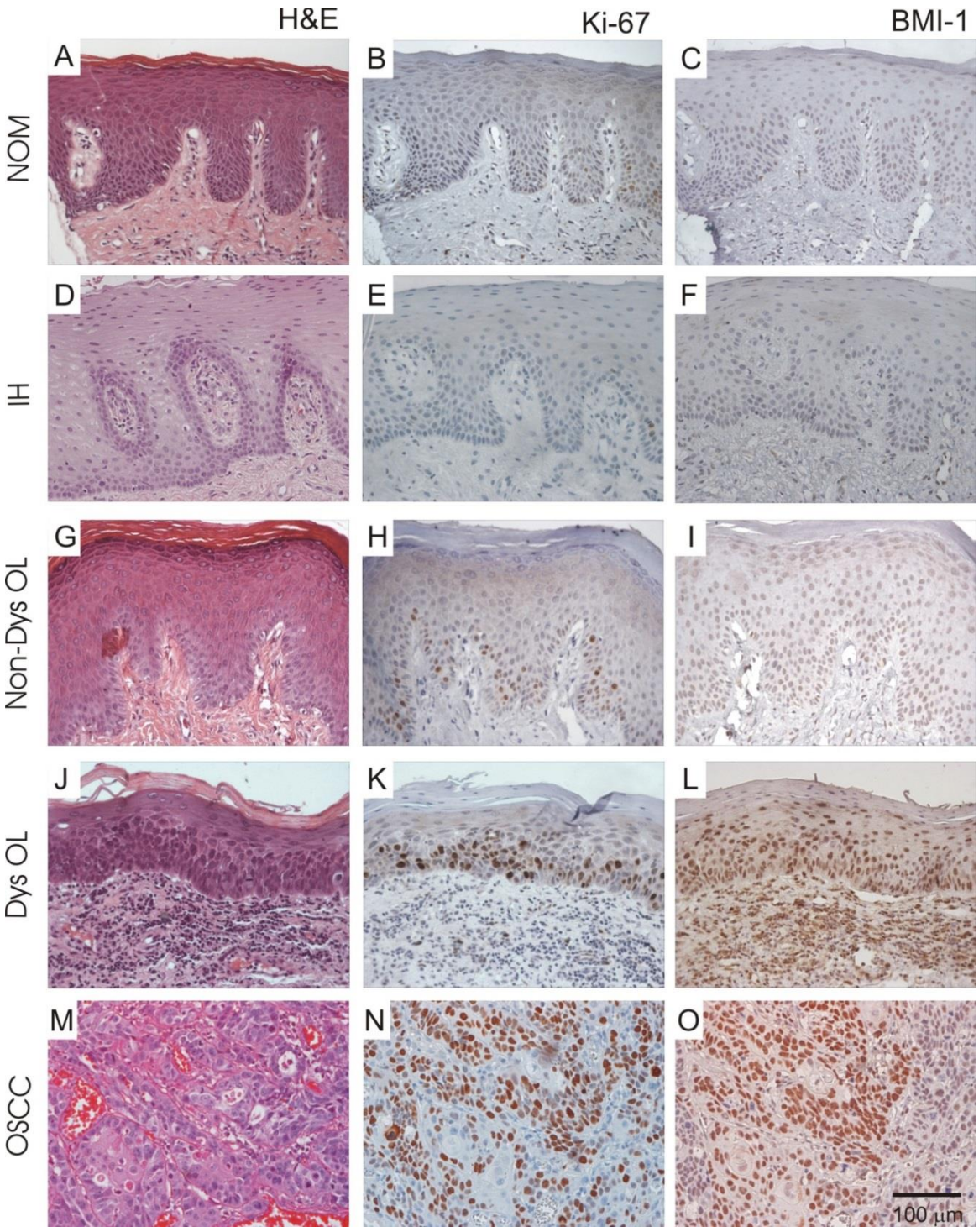
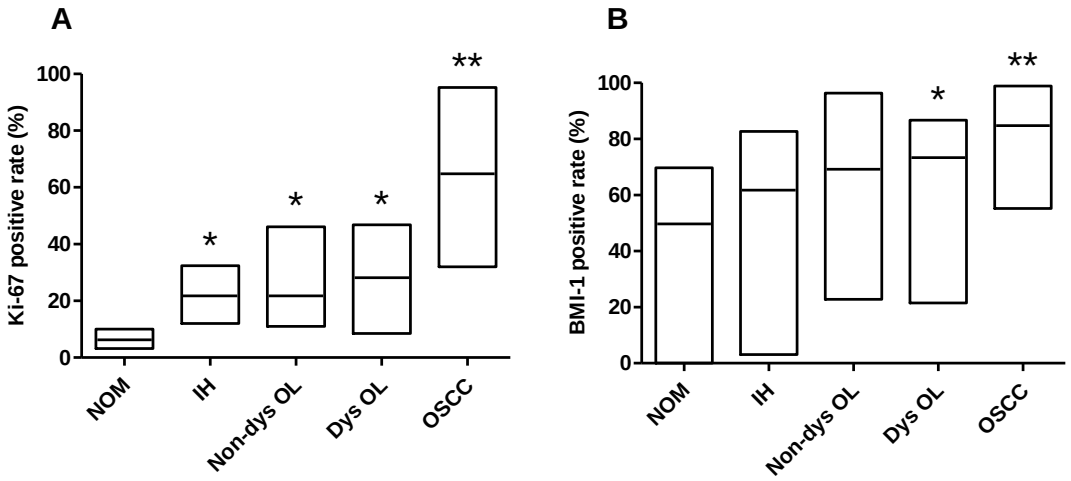


Figure 3. Comparison of immunolabeling for Ki-67 (A) and BMI-1 (B) between normal oral mucosa (NOM), inflammatory hyperplasia (IH), non-dysplastic oral leukoplakia (Non-dys OL), dysplastic oral leukoplakia (Dys OL), and oral squamous cell carcinoma (OSCC). Data are presented as median and minimum-maximum



*p<0.01 x NOM using Kruskal-Wallis test
**p<0.01 x NOM/IH/Nondys OL/Dys OL using Kruskal-Wallis test

Table 3. Percentage of immunopositive cells for Ki-67 in epithelial layers of normal oral mucosa (NOM), inflammatory hyperplasia (IH), and oral leukoplakia (OL).

	NOM	IH	OL		P
			Non-dys	Dys	
Basal layer*	**8.0** [a]	**21.6** [b]	**23.0** [b]	**31.2** [b]	<0.01
	(4.2 - 10.0)	(4.8 - 57.8)	(8.4 - 69.6)	(6.2 - 66.2)	
Parabasal layer*	**10.4** [a]	**36.4** [b]	**37.0** [b]	**37.6** [b]	<0.01
	(4.4 – 22.2)	(11.4 – 59.4)	(16.2 - 63.2)	(11.8 - 69.2)	
Suprabasal layer*	**0.0** [a]	**0.8** [ab]	**4.0** [bc]	**5.0** [c]	<0.01
	(0.0 - 0.8)	(0.0 - 6.4)	(0.0 - 17.6)	(0.0 - 44.8)	

Data are presented as median (minimum-maximum); *Kruskal-Wallis test

Table 4. Percentage of immunopositive cells for BMI-1 in epithelial layers of normal oral mucosa (NOM), inflammatory hyperplasia (IH), and oral leukoplakia (OL).

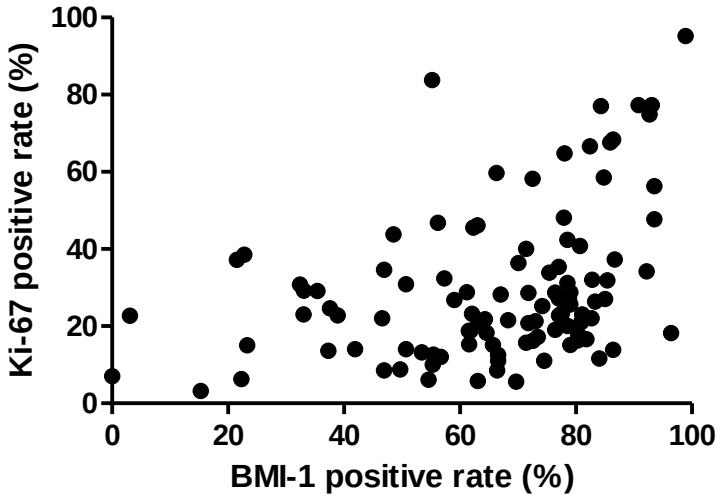
	NOM	IH	OL		p
			Non-dys	Dys	
Basal layer*	**77.4** [a]	**70.6** [a]	**73.9** [a]	**75.8** [a]	0.24
	(0.0 - 89.4)	(9.4 - 87.4)	(20.4 - 96.4)	(33.3 - 91.4)	
Parabasal layer*	**26.0** [a]	**55.8** [ab]	**70.9** [b]	**72.8** [b]	<0.01
	(0.0 – 62.6)	(0.0 – 84.8)	(24.0 - 96.2)	(19.6 - 87.6)	
Suprabasal layer*	**29.5** [a]	**69.0** [ab]	**66.2** [b]	**68.2** [b]	<0.01
	(0.0 – 61.5)	(0.0 – 82.7)	(19.4 - 96.6)	(3.6 - 91.0)	

Data are presented as median (minimum-maximum); *Kruskal-Wallis test

Correlation between BMI-1 and Ki-67

Spearman's correlation coefficients were calculated to determine whether BMI-1 could be implicated in changes in cell proliferation. BMI-1 was directly correlated with Ki-67 (Figure 4, Spearman's correlation, R=0.37, $p < 0.01$).

Figure 4. Correlation between BMI-1 and Ki-67 expression in NOM, IH, OL and OSCC (Spearman correlation coefficient, R=0.37, p<0.01).



Association of Ki-67 and BMI-1 immunolabeling with OL clinical characteristics and histopathological changes

The association between the expression Ki-67 and of BMI-1 with clinical characteristics, histopathological changes, OSCC development and clinical evolution for OL cases is presented in Table 5. Although without statistical significance, higher Ki-67 and BMI-1 expression were demonstrated in relation to lesions located in tongue and floor of the mouth, with epithelial dysplasia, OSCC development and poor clinical evolution. Ki-67 parabasal expression was lower in OL with less than 2 cm in size when compared with higher ones (Student's *t* test, p=0.03, data not shown). In linear regression analysis, the increase of 4.21% in Ki-67 expression in suprabasal layers cells was associated with the degree of dysplasia (95% CI, 3.25–7.12; p=0.03).

Table 5. Clinical and histopathological characteristics in OL cases association the expression of Ki-67 and BMI-1.

	Ki-67(%) Mean(SD)	**p**	**BMI-1(%)** Median(Q1-Q3)	**p**
Location				
Tongue / floor of the mouth	26.8 (8.8)	0.40*	74.2 (63.2-78.6)	0.56**
Other locations	24.6 (9.8)		68.3 (57.6-79.7)	
Clinical type				
Homogeneous	25.8 (9.1)	0.70*	71.6 (52.1-80.1)	0.82**
Non homogeneous	24.7 (10.5)		68.3 (62.3-78.6)	
Presence of epithelial dysplasia				
No	23.5 (08.0)	0.19*	69.2 (53.6-80.0)	0.70**
Yes	26.6 (10.6)		73.4 (60.1-78.6)	
Lesion size				
<2 cm	23.2 (7.9)	0.09*	71.0 (56-9-80.5)	0.51**
≥2 cm	28.0 (10.6)		69.2 (57.5-78.3)	
OSCC development				
Yes	33.1 (9.1)	0.15*	73.4 (34.9-77.8)	0.75**
No	24.5 (9.6)		66.5 (50.7-76.4)	
Clinical evolution				
Good	23.9 (10.8)	0.31*	65.2 (0.7-74.3)	0.52**
Poor	27.1 (8.5)		69.9 (44.9-78.7)	

*Student's t test; **Mann Whitney's test.

Discussion

Oral carcinogenesis is a complex process resulting from various genetic and epigenetic changes. Among them, overexpression of BMI-1 seen in OSCC cells, is thought to relate to an increase in cell proliferation [21], and seems to predict tumorigenesis [26-28]. To the best of our knowledge, the present study is the first one to assess Ki-67 and BMI-1 expression in the same sample of OL [29]. As expected, a increase in those markers in OL cases when compared to NOM. Interestingly, statistically significant changes were observed in early stages of carcinogenesis [27, 28, 30]. Moreover, it was found that BMI-1 immunolabeling levels were directly associated with cell proliferation in epithelium during carcinogenesis [31, 32].

It is well known that one of the main events of carcinogenesis is increase in cell proliferation [33, 34]. The present findings showed an increased percentage of Ki-67 positive cells in Non-dys and Dys OL when compared with NOM. Moreover, cell proliferation was higher in OSCC in relation to NOM, IH and Non-dys and Dys OL. Interestingly, the cell proliferation index was also higher in IH than in NOM. Taking into account that IH has no potential malignant disorder, this cell proliferation increase *per se* should not be interpreted directly as a sign of change toward tumorigenesis.

As found in previous studies, the percentage of Ki-67 positive cells was slightly higher (but not statistically significant) in Dys OL than in Non-dys OL [30, 35]. To improve the evaluation, an additional analysis was performed to evaluate the distribution of Ki-67 positive cells in the different epithelial layers. The basal and parabasal layers are expected to be the sites of normal proliferating cells. In contrast, suprabasal layers comprise the compartment of epithelial maturation in which morphological signs of dysplasia are frequently detected [34, 36]. Our results agree with this assumption because in NOM the proliferating cells were restricted mainly in the basal layer [37, 38]. Another important data was that Ki-67 expression in parabasal cells was significantly higher in OL with or higher than 2 cm in size. The increase in Ki-67 suprabasal expression was associated with the degree of dysplasia and was significantly higher in Dys OL in relation to IH. Thus, the present findings reinforce the data found in the literature, showing that Ki-67 is a biomarker capable of distinguishing NOM from OL and IH, when evaluating the suprabasal layer. Furthermore, assessment of the suprabasal layer showed that Ki-67 expression was

progressively higher according to the degree of epithelial dysplasia [39]. Therefore, the findings shown herein reinforce that Ki-67 expression is a valuable marker as predictor of oral leukoplakia behavior, particularly when suprabasal layer cells are evaluated, reinforcing that the presence of mitosis in the upper half of the epithelium is an important criterion in the morphological analysis [12, 40].

BMI-1 has been involved in the transcriptional repression of Hox genes, and it affects stem cell self-renewal, embryonic development, and proliferation [41, 42]. Our results show that BMI-1 positivity increase was gradual and steady. In NOM, BMI-1 expression was statistically lower when compared with Dys OL and OSCC. These data suggest that BMI-1 expression increases during carcinogenesis. However, the analysis considering BMI-1 immunolabeling without epithelial layers distinction showed no differences between OL (Non-dys and Dys) and IH.

BMI-1 expression in OSCC was higher than in Non-dys and Dys OL. In contrast, Dys OL and OSCC showed elevated BMI-1 immunopositivity compared with NOM. Non-dys OL presented a higher, but not statistically different, positivity of BMI-1 if compared with NOM, when all layers were evaluated together. It is probable that the higher percentage of BMI-1 positive cells in the basal layer of NOM leads to similar medians of immunolabeling when compared with IH and Non-dys OL. These results are in accordance with Kang et al. (2007) who found that an increased BMI-1 expression was associated with dysplastic changes during oral carcinogenesis. These findings might also be explained by the participation of BMI-1 in epithelial-to-mesenchymal transition, as demonstrated in previous studies with breast cancer cells. In addition, overexpression is seen in cancer cells, activates PI3K/AKT signaling pathway and relates to migration and metastasis [43, 44]. Since different events of tissue renewal occur in each epithelial compartment, a complementary analysis assessed each layer separately. A high positivity of BMI-1 was observed in the basal cell layer of NOM, IH, and OL (no statistical difference). This might be explained by the role of this layer in self-renewal. These results suggest that BMI-1 was not only closely associated with premalignant changes but also was present in normal stratified squamous epithelium. This could be explained by the dependence of oral epithelium on stem cells for tissue renewal [45]. Other possible reason could be the association of BMI-1 with E-cadherin and cell-cell junction assembling [43].

BMI-1 immunolabeling was statistically higher in the parabasal and suprabasal layers in Non-dys and Dys OL when compared with NOM. In the normal oral

epithelium, the expression of stem cells is expected to be down-regulated and barely detected in the upper layers of the epithelium. However, in the dysplastic epithelium, maintenance of BMI-1 expression above the basal cell layer may provide an anti-differentiation effect and improve proliferative capacity. These events could lead to architectural disturbances that become evident by the presence of epithelial dysplasia [46]. The higher proportion of these cells in the upper layers could be the reason for the morphological finding of loss of stratification, a remarkable criterion for the presence of epithelial dysplasia [22]. Taking into account the abovementioned considerations, studies on leukoplakia should consider the epithelial layers separately.

Other important result was the significant correlation found between Ki-67 and BMI-1 immunolabeling. This may be attributed to the role of BMI-1 in the regulation of cell proliferation by suppressing ink4a expression, a locus that triggers senescence in human somatic cells [47]. Therefore, the switch between differentiation or epithelial-mesenchymal transition, which depends on genetic and epigenetic events, is modulated by the control of cell growth, survival, angiogenesis, and motility. The balance of these cross-talking signaling pathways is the base for acquisition of a malignant phenotype and progression to OSCC. Despite the large number of studies on this, the knowledge about individual factors needs to be improved in order to develop strategies for cancer prevention [48].

Liu et al. (2012) found that BMI-1 expression was associated with the development of oral cancer in patients with OL, suggesting that BMI-1 could be used as a predictor of OL transformation. On that study, approximately 13% of 135 OL patients demonstrating BMI-1 positivity developed OSCC, compared with 10.3% patients negative for BMI-1. In our study, 10.0% of 40 patients with OL who were followed-up developed OSCC. In those patients, the presence of severe epithelial dysplasia was found to be a predictor for OSCC development. The same group of patients displayed increase (not statisticaly significant) in Ki-67 and BMI-1 expression. Regarding location, three of four cases were located in the tongue/floor of the mouth, reinforcing that OL in those site present a more aggressive behavior [25].

To the best of our knowledge, this study is the first to evaluate Ki-67 and BMI-1 in the same sample of OL. Based on our results, it is possible to suggest that cell proliferation and changes toward epithelial-to-mesenchymal transition are related events during carcinogenesis. The Ki-67 data reinforced that cell proliferation

increases gradually from NOM to OL and later on to OSCC. Importantly, all OL cases displayed BMI-1, indicating changes and differentiation profile, which was not influenced by the presence of epithelial dysplasia. This finding indicates that higher expression of BMI-1 occurs early during oral carcinogenesis and that it may be used as a marker of preneoplastic oral lesions. The present findings indicate that both Ki-67 and BMI-1 have abnormal expression during carcinogenesis and that they participate in the beginning and progression of OSCC.

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4. CONSIDERAÇÕES FINAIS

A leucoplasia é a lesão mais frequente dentre as desordens potencialmente malignas de boca, com prevalência estimada entre 0,42 e 5% (3-5). A taxa de transformação maligna dessa lesão varia 0,13-17,5% (21, 22). Ainda que muitos estudos tenham sido publicados a respeito desta lesão, o seu prognóstico ainda permanece em discussão. A utilização de biomarcadores tem sido uma ferramenta importante para tentar identificar pacientes com leucoplasia com maior risco de desenvolver câncer bucal. Além disso, esta estratégia pode ajudar na detecção dos primeiros estágios da transformação maligna. Ao longo da carcinogênese, o desequilíbrio entre proliferação celular e apoptose parece ser uma etapa importante (46-48). Diversos estudos têm mostrado um aumento da proliferação celular progressivo ao longo da carcinogênese (41,49,50). Sendo o Ki-67 um marcador de proliferação celular e o BMI-1 relacionado à transição epitélio-mesênquima, foi avaliada a expressão desses dois biomarcadores em leucoplasia bucal. Nesse trabalho, utilizamos mucosa normal, hiperplasia inflamatória e carcinoma espinocelular como grupos de comparação para avaliação da imunomarcação em lesões patológicas que reproduzem estágios da carcinogênese. Os resultados possibilitaram observar que há um aumento progressivo da expressão desses marcadores na carcinogênese. Além disso, concluímos que é de suma importância avaliar a expressão desses marcadores com base em uma análise diferencial em três camadas do epitélio (basal, parabasal e suprabasal), método de análise que já havia sido utilizado em estudos prévios (36, 37, 40). Essa abordagem justifica-se à medida que as camadas do epitélio apresentam diferentes padrões de normalidade na porcentagem de células positivas. A partir dessa constatação, entende-se que as células da camada basal apresentaram alta positividade em mucosa normal, principalmente para BMI-1, devido à função de autorrenovação das células dessa camada (67,68). Alterações morfológicas e de maturação são observadas nas camadas intermediárias do epitélio (69). Por essas razões, é importante considerar as camadas epiteliais separadamente em estudos com leucoplasia.

Os resultados desse estudo sugerem que a proliferação celular e alterações na transição epitélio-mesênquima são eventos relacionados durante a carcinogênese. Além disso, o aumento da expressão de BMI-1 ocorre nos estágios precoces da carcinogênese oral e pode ser utilizado para o estudo do

comportamento desordens potencialmente malignas. Esses achados indicam que Ki-67 e BMI-1 têm expressão anormal e diferenciada ao longo da carcinogênese.

Apesar dos inúmeros estudos publicados até o presente momento, a compreensão do comportamento da leucoplasia bucal é bastante limitada. O entendimento do papel do BMI-1 na carcinogênese tem potencial para contribuir no estabelecimento de alternativas terapêuticas que, no futuro, podem favorecer que leucoplasias sejam interceptadas, prevenindo a transformação maligna. Nesse sentido, mais estudos utilizando outros marcadores podem ser úteis.

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