The value of Phosphohistone H3 as a cell proliferation marker in oral squamous cell carcinoma. A comparative study with Ki-67 and the mitotic activity index

Natalia Tancredi-Cueto 1, Gabriela Vigil-Bastitta 2, Ronell Bologna-Molina 3, Verónica Beovide Cortegoso 4

1 DMD. Master Student (Dentistry). Oral Histopathology Laboratory, Faculty of Dentistry, University of the Republic, Uruguay
2 DMD. Master Student (Dentistry). Molecular Pathology Area, Faculty of Dentistry, University of the Republic, Uruguay
3 PhD. Molecular Pathology Area, Faculty of Dentistry, University of the Republic, Uruguay
4 PhD. Oral Histopathology Laboratory, Faculty of Dentistry, University of the Republic, Uruguay

Correspondence:
Oral Histopathology Laboratory, Faculty of Dentistry
University of the Republic, Uruguay
nataliatancred77@gmail.com

Received: 23/03/2022
Accepted: 04/07/2022

Abstract
Background: The Phosphohistone H3 (PHH3) antibody is recognized as a biomarker of cell proliferation, specific for cells in mitosis, of prognostic value in different malignant neoplasms, however it has been poorly studied in oral squamous cell carcinoma (OSCC). The main objective of this study was to evaluate the immunoperoxidase expression of the PHH3 in the OSCC, through the correlation with the immunoperoxidase expression of Ki-67, the mitotic activity index (MAI), histological grading, clinical-morphological parameters and the rate of survival.
Material and Methods: The study sample consisted of 62 cases of OSCC diagnosed in the Pathological Anatomy Laboratory of the Faculty of Dentistry, University of the Republic (Uruguay). In each of them, an immunohistochemical technique was performed for Ki-67 and PHH3 (serine 10) antibodies. Image J software was used for the MAI and biomarker quantification, defining the percentage of positivity and mitotic figures per 1000 tumor cells. Results: a significant association was obtained between the expression of PHH3 (p 0.016) and MAI (p 0.031) with survival time. However, no similar relationship was found with Ki-67 (p 0.295). Although it was confirmed a statistical association between histological grade and Ki-67 immunoperoxidase (p 0.004), PHH3 did not show a similar relationship (p 0.564).
Conclusions: It was confirmed the role of the PHH3 antibody as a biomarker of mitotic figures in OSCC and as a potential marker of cell proliferation. It is noteworthy that this is one of the first works that evaluates a possible relationship between the expression of this antibody and survival in OSCC.

Key words: Oral squamous cell carcinoma, phosphohistone H3, Ki-67, cell proliferation.

doi:10.4317/medoral.25420
Tancredi-Cueto N, Vigil-Bastitta G, Bologna-Molina R, Beovide-Cortegoso V. The value of Phosphohistone H3 as a cell proliferation marker in oral squamous cell carcinoma. A comparative study with Ki-67 and the mitotic activity index. Med Oral Patol Oral Cir Bucal. 2022 Sep 1;27 (5):e444-51.

Article Number:25420           http://www.medicinaoral.com/
© Medicina Oral S. L. C.I.F. B 96893836 - pISSN 1698-4447 - eISSN: 1698-6946
E-mail: medicina@medicinaoral.com
Indexed in:
Science Citation Index Expanded
Journal Citation Reports
Index Medicus, MEDLINE, PubMed
Scopus, Embase and Eincare
Indice Médico Español

Journal section: Oral Cancer and Potentially malignant disorders
Publication Types: Research
Introduction

Oral squamous cell carcinoma (OSCC) represents the most common cancer of head and neck region (1,2). It is characterized by aggressive biological behavior and an unfavorable prognosis (3,4). In order to improve the low survival rate of patients with OSCC, an important line of research is focused on the identification of molecular biomarkers of prognostic value that contribute to the selection of the most appropriate therapeutic plan (3,5,6).

Cell proliferation is an essential biological process, key in the growth and maintenance of tissue homeostasis, whose loss of control plays a fundamental role in the development of malignant neoplasms (4,6). In fact, in several types of human cancer, the evaluation of cell proliferation is considered an important histological parameter (7). For its assessment, different molecular antigens have been identified, which, when expressed by cells in active proliferation, can be used as predictive biomarkers of sustained proliferation (8). MAI and Ki-67 immunoexpression are the most widely used methods to determine tumor proliferative capacity, however, both methods are objected to present significant intra and interobserver variability (9).

MAI represents the oldest method for determining the proliferative capacity of malignant neoplasms (10,11). It is obtained by counting normal and atypical mitotic figures (MF) in a cell population of known number (10). The Ki-67 antigen is a non-histone nuclear protein present in all active phases of the cell cycle (G1, S, G2 and M), being absent only in resting cells (G0) (4,9,12). The Ki-67 antibody by immunohistochemical techniques (IHC) allows the identification of the Ki-67 protein in tissue samples (4,12,13). Actively proliferating cells immunoexpress this antibody, varying in intensity and location, depending on the phase of the cell cycle and the history of each individual cell (14,15). The quantification of Ki-67 immunoexpression is performed through the labeling index, defined as the percentage of positive cells in a cell population of known number (3,4,12).

The PHH3 antibody is recognized as a biomarker of cell proliferation, specific for cells in mitosis, by identifying phosphorylated histone H3 by IHC (9,11,16). Histone H3 is one of the five different types of histone proteins that are part of nucleosomes, whose phosphorylation at the serine 10 and 28 level, determines the compaction of chromatin during cell division and from this event the cell is enabled to enter the M phase of the cell cycle (11,16,17). Histone H3 phosphorylation is initiated non-randomly in pericentromic heterochromatin in late G2 phase and as mitosis progresses, it spreads throughout the chromosome, completing in late prophase and continuing through metaphase (11,16). In anaphase, the dephosphorylation process of histone H3 begins, which ends in early telophase (9,11,16). The PHH3 antibody is characterized by presenting a clear and well contrasted immunostaining, limited to cells that are in the M phase of the cell cycle, while interphase cells do not express it or do so minimally (9,11,18). Although it has been recognized as a prognostic marker in multiple types of cancer, it has been scarcely analyzed in OSCC and, in particular, its relationship with survival rate has not been the subject of research until now (16,19).

The main objective of this study was to evaluate the immunoexpression of PHH3 in OSCC, through the correlation with the immunoexpression of Ki-67, MAI, histological grading, clinical-morphological parameters and the rate of survival.

Material and Methods

The study sample consisted of 62 cases of OSCC, corresponding mostly to incisional biopsies diagnosed in the Pathological Anatomy Laboratory of the University of the Republic (UdelaR) School of Dentistry, in the period 2007-2015. The clinical pathological records of each of the cases were reviewed, recording the variables corresponding to gender, age, topography, date of pathological diagnosis and histopathological diagnosis. Survival was defined as the time elapsed between the initial histopathological diagnosis and death from cancer. The histopathological diagnosis of each of the cases was made according to the WHO classification (2017) for OSCC, in well, moderately and poorly differentiated (1,2).

Hematoxylin-eosin (H-E) and IHC histological slides were digitized with the Motic VM 3.0 Digital Slide Scanning System for image acquisition and processing. Motic VM 3.0 Motic Digital Slide Assistant software (version 1.0.7.46, Copyright Motic China Group Co., Ltd.2017) was used for its analysis. Cell quantification was performed with the Image J software manual counting tool (1.52v, Wayne Rasband, National Institutes of Health, USA). For the calculation of the MAI, in the H-E slides, normal and atypical MF present in 1000 tumor cells were counted. The identification of the MF was carried out according to the morphological characteristics described by Van Diest et al, recognizing as atypical mitosis, those multipolar, annular, asymmetric and bridge in anaphase (20,21).

Immunohistochemical processing: from the blocks of tissue fixed in formalin and embedded in paraffin, two sections of 4 μm thickness were obtained. To unmask the antigenic epitopes, recovery was performed with oxidation enzymes were blocked with 0.9 % hydrogen peroxide (pH 6.2) (Borg Decloaker, RTU; Biocare Medical) in a microwave pressure cooker at maximum power for about 5 minutes. Endogenous peroxidases were blocked with 0.9 % hydrogen peroxide for 5 minutes. Tissue sections were incubated with the primary antibodies Ki 67 (monoclonal; 1:100 dilution, clone MIB-1, Dako, Glostrup, Denmark) and PHH3 (Serine 10) (monoclonal; 1:100 dilution, Novus Biologica...
Prior to the IHC evaluation, interobserver calibration was performed between two pathologists, who previously agreed on the morphological criteria necessary for the identification of MF and positive cells for biomarkers. The observers carried out their quantifications independently, without knowing the figures established by each other. The interclass correlation coefficient (ICC) was used to calculate the degree of interobserver agreement (9).

Statistical analysis: in the considered markers comparison, descriptive statistics (mean and standard deviation) were used for categorical variables, and frequency
distribution for continuous variables. To compare the expression of the markers according to sex, age, location and histological grade, when it came to two groups, the comparison of means based on the student's t-test for independent samples was used. Likewise, in cases where three or more groups were compared, the analysis of variance (ANOVA) model was used. In cases where a significant association was identified, multiple comparisons were carried out considering the Tukey test. Additionally, scatter diagrams were made to investigate the degree of linear correlation between the three markers, calculating in each case, the Pearson linear correlation coefficient. Survival analysis was carried out using the Kaplan-Meier survival curves. The association of patient survival was analyzed according to the three markers considered, in a multivariate analysis through the Cox proportional hazards model, adjusting the results by sex, age, grouped location and histological grade. While their comparisons were made using the Cox model. All tests were carried out with a significance level of 5%.

Results
- Descriptive analysis of the clinical-pathological variables of OSCC: of the 62 cases, the 64.6% corresponded to men and the 59.7% were older than 65 years, the 43.5% were located in others (under this term the located cases were grouped in gingiva, alveolar ridge and extensions to the retromolar trigone and cheek), 25.8 % in tongue, 21.0 % in palate and 9.7 % in the mouth’s floor. Regarding histological grading, 61.3 % corresponded to moderately differentiated OSCC (Grade 2), 27.4 % to well-differentiated OSCC (Grade 1), and only 1.3 % were classified as poorly differentiated OSCC (Grade 3) (Table 1).
- Association between clinical-pathological characteristics and biomarkers Ki-67 and PHH3: a statistically significant relationship was found between the histological grade of OSCC and the immunoexpression of Ki-67 ($p < 0.004$) (Table 1). Thus, in the well differentiated OSCC, a lower expression of Ki 67 was observed (mean 28.97), rising in the moderately differentiated OSCC (mean 41.27) and reaching the highest expression in the poorly differentiated OSCC (mean 43.37). However, a similar relationship was not found with the PHH3 biomarker ($p = 0.564$). Neither could a statistically significant correlation be demonstrated between the biomarkers studied and the independent variables, sex, age and location (Table 1).
- Expression patterns of Ki-67 and PHH3: When correlating the Ki-67 and PHH3 variables, a slight significant relationship was found ($p = 0.041$) (Fig. 3). In general, the degree of expression of the PHH3 antibody was markedly lower than that of Ki-67, with a mean of 1.34 (SD 14.44) and 38.14 (SD 14.44), respectively. Additionally, the PHH3 antibody was characterized because in addition to presenting a narrow range of immunostaining (range = 3.1; lower limit = 0.2 and upper limit = 3.3), the intensity of the staining was strong and positively correlated with the morphology of the nuclei according to the different stages of mitosis. In contrast, the Ki-67 antibody demonstrated a wide range of expression (range = 54.2, lower limit = 8.5 and upper limit = 62.7) with variable staining patterns and intensity (Fig. 4).

Table 1: Association between the clinicopathological characteristics of OSCC and the dependent variables: MAI, immunoexpression of Ki-67 and PHH3.

| Global          | n   | Ki-67        | PHH3         | MAI         |
|-----------------|-----|--------------|--------------|-------------|
|                 |     | M 38.14 (SD 14.44) | M 1.34 (SD 0.62) | M 0.97 (SD 0.55) |
| **Sex**         |     |              |              |             |
| Female          | 23  | 39.31 16.75  | 1.31 0.59    | 1.04 0.43   |
| Male            | 39  | 37.45 13.08  | 1.36 0.65    | 0.93 0.61   |
| **Age**         |     |              |              |             |
| Under 65        | 25  | 39.31 15.33  | 1.33 0.67    | 0.98 0.56   |
| Over 65         | 37  | 36.40 13.14  | 1.36 0.56    | 0.96 0.55   |
| **Grouped Localization** |     |              |              |             |
| Tongue          | 16  | 35.34 14.75  | 1.22 0.59    | 0.81 0.35   |
| Others          | 27  | 39.10 15.56  | 1.47 0.71    | 1.01 0.54   |
| Palate          | 13  | 40.35 11.87  | 1.21 0.47    | 1.09 0.69   |
| Mouth’s floor   | 6   | 36.48 15.78  | 1.35 0.55    | 1.93 0.73   |
| **Histological grade** |     |              |              |             |
| 1               | 17  | 28.97 a 13.72 | 1.19 0.63    | 1.00 0.54   |
| 2               | 38  | 41.27 b 12.74 | 1.43 0.62    | 0.91 0.52   |
| 3               | 7   | 43.37ab 16.87 | 1.21 0.61    | 1.21 0.70   |

Abbreviations: M (mean), SD (standard deviation). According to Tukey’s test: a, b, ab, indicate different significant groups. Significant values are shown in bold. * $p \leq 0.05$. 
- Survival analysis: the event of death was observed in 48 patients, while 14 patients reached the end of the follow-up period. The survival curve was constructed using the Kaplan-Meier procedure, from which it was estimated that the median overall survival time was 1.51 years (0.92; 2.69) and the survival rate at five years was 27 % (0.18; 0.41) (Fig. 3). Likewise, the study of the possible association between each variable (Ki-67, PHH3 and MAI) and patient survival was carried out in a bivariate and multivariate manner. From this analysis, in its two forms, it was possible to observe a certain significant association between survival time and PHH3 expression ($p < 0.016$) (Fig. 3). A significant and similar relationship is also observed with MAI ($p < 0.031$) (Fig. 3), confirming that, at higher values of this, as well as of the immunoexpression of PHH3, the shorter the survival time. In contrast, for KI-67, no statistically significant association was found ($p > 0.295$) (Fig. 3). Table 2 shows the results obtained.

![Fig. 3](image)

- Fig. 3: A. Scatter plot showing the existence of a significant but low correlation between PHH3 and Ki-67 ($r = 0.259 / p = 0.041$). B. Graphic representation showing no significant correlation between Ki-67 and MAI ($r = 0.167 / p = 0.194$). C. As shown in this diagram, a significant correlation was found between PHH3 and MAI ($r = 0.450 / p < 0.001$). D. Kaplan-Meier curve of overall survival. E. Association between survival and PHH3 expression. High expression of PHH3 was significantly associated with a shorter survival period. F. Association between survival and MAI. The increase in MAI was significantly correlated with a shorter survival period. G. Association between survival and Ki-67 expression. High expression of Ki-67 was not significantly associated with a shorter survival period. The Kaplan-Meier E, F, and G curves arise from the Cox proportional hazards model. They are adjusted curves for the different values of the variables.

![Fig. 4](image)

- Fig. 4: Comparison of the same tumor area in slides stained with H-E and IHC for Ki-67 and PHH3. A. Sheet of H-E where two MF can be identified. B. IHC slide for Ki-67, where several positive cells with nuclear staining of variable intensity are observed. C. IHC slide for PHH3, in which a lower number of positive cells are visualized compared to the IHC slides for Ki-67, identifying four MF that immunoexpress the PHH3 antibody. 20x magnification (A-C).
The value of PHH3 as a cell proliferation marker in OSCC

When analyzing the relationship between biomarkers and MAI, not enough evidence was found to indicate a significant correlation with Ki-67 \((p = 0.194)\) (Fig. 3). In contrast, based on the calculated correlation coefficient \((r = 0.450)\) with a \(p\)-value < 0.001, a statistically significant association was observed between MAI and PHH3 (Fig. 3). Likewise, the PHH3 antibody allowed us to more easily identify the positive MF and the fields with the highest mitotic density, thus being able to confirm its role as a specific marker of mitosis. In fact, as shown in Table 3, in 44 cases, the number of mitosis identified by IHC for PHH3 was higher than that obtained based on the recognition of its morphological characteristics in H-E stained slides.

Table 2: Bivariate and multivariate analysis of the association between Ki-67, PHH3 and MAI and patient survival.

| Parameter     | Bivariate | Multivariate |
|---------------|-----------|--------------|
|               | N         | HR    | \(p\)  | HR    | \(p\)  |
| Ki-67         | 1,010 (0.991; 1.030) | 0.295 | 1,001 (0.983; 1.128) | 0.605 |
| PHH3          | 1,061 (1.011; 1.113) | 0.016* | 1,052 (1.002; 1.105) | 0.042* |
| MAI           | 1,056 (1.005; 1.110) | 0.031* | 1,054 (1.001; 1.109) | 0.045* |

Abbreviation: HR (hazard ratio). Note: The values that appear in parentheses attached to the HR correspond to the 95% confidence interval. Significant values are shown in bold. * \(p \leq 0.05\).

In this study, the median overall survival time was only 1.51 years (0.92; 2.69) and the five-year survival rate, without considering the stage of the disease, was 27 %. (0.18, 0.41). The low survival rate in our study could be partly because of two reasons. In first place, in Uruguay, the OSCC diagnosis is made in advanced stages, as established in the few publications available (23,24). Second, the sample selection was non-probabilistic for convenience.

Discussion

The evaluation of cell proliferation in cancer is considered an important histological parameter for defining the biological behavior of the tumor and for determining the individualized prognosis for each patient (7). However, in OSCC, evaluation of tumor proliferation is not part of the current staging system (2). Nevertheless, it constitutes a line of research, like so many others, justified in part by the poor survival rate of diagnosed patients, even in early stages of the disease (4,13). In fact, in this study, the median overall survival time was only 1.51 years (0.92; 2.69) and the five-year survival rate, without considering the stage of the disease, was 27 %. (0.18, 0.41). The low survival rate in our study could be partly because of two reasons. In first place, in Uruguay, the OSCC diagnosis is made in advanced stages, as established in the few publications available (23,24). Second, the sample selection was non-probabilistic for convenience.

The PHH3 antibody, recognized as a biomarker of cell proliferation and specific for cells in mitosis, has been little studied in comparison with other biomarkers of cell proliferation (25). In contrast, in OSCC, unlike the PHH3 antibody, one of the cell proliferation biomarkers that has been widely studied is Ki-67 (4,13,22,26). However, studies on the value of the expression of this biomarker in determining the survival of patients with OSCC have shown contradictory results (3,15). In fact, there was not a statistically significant association between Ki-67 expression and patient survival \((p = 0.295)\). Along the same lines, the results obtained by Brockton et al. and Gonzales Moles et al. (27,28). However, it is important to note that recently published research, such as that by Gadball et al. and Jing et al. recognize Ki-67 immunohistopstaining as a reliable prognostic factor in OSCC (4,22). These authors found a significant relationship between the degree of Ki 67 immunostaining and survival, after studying Ki 67 expression in large cohorts of 217 and 298 OSCC cases, respectively (4,22). Unlike Ki-67, in this study it was demonstrated a significant relationship between the expression of PHH3 and the survival time of patients with OSCC \((p = 0.016)\). Although it was previously established in invasive breast and urogenital cancer, it wasn’t found any work in the literature that had previously studied this relationship in OSCC (9,25). It was also observed a significant relationship between the expression of Ki-67 and PHH3 \((p = 0.041)\); expected since both are biomarkers of cell proliferation expressed by the fraction of cells that are actively passing through the cell cycle. This association was described in breast cancer by Kim et al. and in follicular lymphoma by Bedekovics et al.(9,19). In addition, as previously de-
scribed, it was able to verify a significant relationship between the degree of histological differentiation of OSCC and Ki-67 immunoeexpression (p < 0.004) which supports the potential usefulness of this biomarker in the histopathological classification of OSCC (4,13,22,28). Thus, the least differentiated OSCCs at the histological level are those with the highest number of Ki-67 positive cells (4,13,22,28). However, there was not a similar relationship with the biomarker PHH3 (p 0.564).

When compared the immunostaining patterns of the studied biomarkers, in the Ki-67 slides, nuclear immunostaining was shown with a wide range of intensities, a factor that contributes to low reproducibility (9-11,29). On the other hand, PHH3 showed small variations in the intensity of expression and since only those cells with a strong brown stained nucleus and normal or atypical MF morphology should be recognized as positive, in general terms it was easier to identify and quantify (9,10,19). It is important to specify that, as has been observed by other authors, in some cases, the PHH3 biomarker was expressed by cells whose nucleus preserved the nuclear membrane intact and did not present MF morphology, the latter should not be considered in the quantification, for be cells in G2 phase where phosphorylation of histone H3 begins and not cells in mitosis, which do present complete phosphorylation of histone H3 (10,19) (Fig. 2). In addition, as has been previously described in follicular lymphoma and breast cancer, there was a statistically significant association between MAI and PHH3 (p < 0.001), expected because the PHH3 antibody is a specific IHC biomarker of MF and because it was used the same quantification criteria, both in the H-E slides for MAI and in those for IHC (9,18,19,21).

The main limitations of the study are the sample size, not including the tumor invasion front as an evaluation parameter because most of the biopsies were incisional, and not having the TNM stage of the cases analyzed.

Conclusions

The PHH3, is a biomarker of cell proliferation specific to cells in mitosis, with respect to Ki-67, has been under-investigated in the literature. In this work, a significant relationship was demonstrated between the immunoeexpression of the PHH3 and the survival of patients with OSCC. A significant association of MAI with survival time was also observed. Regarding the Ki-67, as was previously described in the literature, a positive association was confirmed between the degree of histological differentiation of the OSCC and the marker’s positive immunoeexpression. Based on the results obtained, it is important to continue investigating the PHH3 proliferation biomarker in OSCC, with a uniformly standardized IHC protocol and in a large cohort of cases.

References

1. Speight PM, Farthing PM. The pathology of oral cancer. Br Dent J. 2018;225:841-7.
2. Almangush A, Måkitie AA, Triantafyllou A, de Bree R, Strojan P, Rinaldo A, et al. Staging and grading of oral squamous cell carcinoma: An update. Oral Oncol. 2020;107:104799.
3. Lopes VKM, de Jesus AS, de Souza LL de, Miyahara LAN, Guimarães DM, Pontes HAR, et al. Ki-67 protein predicts survival in oral squamous carcinoma cells: an immunohistochemical study. Braz Oral Res. 2017;31:e66.
4. Gadgil AR, Sarode SC, Chaudhary MS, Gondvirkar SM, Tekade SA, Yuwanati M, et al. Ki67 labelling index predicts clinical outcome and survival in oral squamous cell carcinoma. J Appl Oral Sci. 2021;29:1-10.
5. Gioacchini FM, Alicandri-Ciufelli M, Kaleci S, Magliulo G, Presutti L, Re M. The prognostic value of cyclin D1 expression in head and neck squamous cell carcinoma. Eur Arch Oto-Rhino-Laryngol. 2016;273:801-9.
6. Maragon Junior H, Leão PLR, Melo VVM, Caixeta ÂB, Souza PEA, de Aguiar MCF, et al. Cell proliferation is associated with intensity of tumor budding in oral squamous cell carcinoma. J Oral Pathol Med. 2018;47:128-35.
7. Chitra NS, Boaz K, Srikant N, Lewis AJ, Sneha KS. Pattern-correlated mitotic activity index (PMAI): A novel prognosticator of oral squamous cell carcinoma. Turk Patoloji Derg. 2020;36:31-8.
8. Blatt S, Krüger M, Ziebart T, Sagheb K, Schiehnitz E, Goetze E, et al. Biomarkers in diagnosis and therapy of oral squamous cell carcinoma: A review of the literature. J Cranio-Maxillofacial Surg. 2015;47:722-30.
9. Kim JY, Sook Jeong H, Chung T, Kim M, Hee Lee J, Hee Jung W, et al. The value of phosphohistone H3 as a proliferation marker for evaluating invasive breast cancers: A comparative study with Ki67. Oncotarget. 2017;8:65064-76.
10. van Steenhoen JEC, Kuijser A, Kornegoor R, van Leeuwen G, van Gorp J, van Dalen T, et al. Assessment of tumour proliferation by use of the mitotic activity index, and Ki67 and phosphohistone H3 expression, in early-stage luminal breast cancer. Histopathology. 2020;77:579-87.
11. Elmaci I, Altnoz MA, Sari R, Bolukbasi FH. Phosphorylated Histone H3 (PHH3) as a Novel Cell Proliferation Marker and Prognosticator for Meningeal Tumors: A Short Review. Appl Immunohistochem Mol Morphol. 2018;26:627-31.
12. Dias EP, Oliveira NSC, Serra-Canpos AO, da Silva AKF, da Silva LE, Cunha KS. A novel evaluation method for Ki-67 immunostaining in paraffin-embedded tissues. Virchows Arch. 2021;479:121-31.
13. Takkem A, Barakat C, Zakaraia S, Zaid K, Najmeh J, Ayoub M, et al. Ki-67 prognostic value in different histological grades of oral epithelial dysplasia and oral squamous cell carcinoma. Asian Pacific J Cancer Prev. 2018;19:3279-86.
14. Sales Gil R, Vagnarelli P, Ki-67: More Hidden behind a ‘Classic Proliferation Marker’. Trends Biochem Sci. 2018;43:747-8.
15. Xie S, Liu Y, Qiao X, Hua RX, Wang K, Shan XF, et al. What is the prognostic significance of Ki-67 positivity in oral squamous cell carcinoma?. J Cancer. 2016;7:758 67.
16. Lai CPT, Yeong JPS, Tan A Sen, Ong CHC, Lee B, Lim JCT, et al. Evaluation of phospho-histone H3 in Asian triple-negative breast cancer using multiplex immunofluorescence. Breast Cancer Res Treat. 2019;178:295-305.
17. Yang H, Jin X, Dan H, Chen Q. Histone modifications in oral squamous cell carcinoma and oral potentially malignant disorders. Oral Dis. 2020;26:719-32.
18. Khieu ML, Broadwater DR, Aden JK, Coviello JM, Lynch DT, Hall JM. The Utility of Phosphohistone H3 (PHH3) in Follicular Lymphoma Grading: A Comparative Study with Ki-67 and H&E Mitotic Count. Am J Clin Pathol. 2019;151:542-50.
19. Bedekovics J, Irsai G, Hegyi K, Beke L, Krenács L, Gergely L, et al. Mitotic Index Determined by Phosphohistone H3 Immunohis-
tochemistry for Precise Grading in Follicular Lymphoma. Appl Immunohistochem Mol Pathol. 2018;26:579-85.
20. Van Diest PJ, Brugal G, Baak JPA. Proliferation markers in tumours: Interpretation and clinical value. J Clin Pathol. 1998;51:716-24.
21. Ohashi R, Namimatsu S, Sakatani T, Naito Z, Takei H, Shimizu A. Prognostic utility of atypical mitoses in patients with breast cancer: A comparative study with Ki67 and phosphohistone H3. J Surg Oncol. 2018;118:557-67.
22. Jing YUE, Zhou Q, Zhu H, Zhang YE, Song Y, Zhang X. Ki - 67 is an independent prognostic marker for the recurrence and relapse of oral squamous cell carcinoma. Oncol Lett. 2019;17:974-80.
23. Cortegoso AVB, Laureano NK, Silva AD da, Danilecviz CK, Magnusson AS, Visioli F, et al. Cell proliferation markers at the invasive tumor front of oral squamous cell carcinoma: comparative analysis in relation to clinicopathological parameters of patients. J Appl Oral Sci. 2017;25:318-23.
24. Oliveira ML, Wagner V, Sant'ana Filho M, Carrard V, Hugo F, Martins M. A 10-year analysis of the oral squamous cell carcinoma profile in patients from public health centers in Uruguay. Braz Oral Res. 2015;29:1-8.
25. Hao Q, Dai C, Deng Y, Xu P, Tian T, Lin S, et al. Pooling analysis on prognostic value of PHH3 expression in cancer patients. Cancer Manag Res. 2018;10:2279-88.
26. Bhuyan L, Sarangi S, Das BK, Das SN, Nayak S. Proliferative index in invasive tumor front of oral squamous cell carcinoma: A potential prognostic indicator. J Contemp Dent Pract. 2018;19:170-6.
27. Brockton NT, Lohavanichbutr P, Enwere EK, Upton MP, Kornaga EN, Nakoneshny SC, et al. Impact of tumoral carbonic anhydrase IX and Ki - 67 expression on survival in oral squamous cell carcinoma patients. Oncol Lett. 2017;14:5434-42.
28. Gonzalez-Moles MA, Ruiz-Avila I, Gil-Montoya JA, Esteban F, Bravo M. Analysis of Ki-67 expression in oral squamous cell carcinoma: Why Ki-67 is not a prognostic indicator. Oral Oncol. 2010;46:525-30.
29. Lea D, Gudlaugsson EG, Skaland I, Lillesand M, Søreide K, Søreide JA. Digital Image Analysis of the Proliferation Markers Ki67 and Phosphohistone H3 in Gastroenteropancreatic Neuroendocrine Neoplasms: Accuracy of Grading Compared with Routine Manual Hot Spot Evaluation of the Ki67 Index. Appl Immunohistochem Mol Pathol. 2021;29:499-505.

Funding
This project was financed by "Grupos i+d" Comisión Sectorial de Investigación Científica (CSIC) 881880 and funds from Santander Bank, obtained in the research project contest, carried out within the framework of the 90 years of the Faculty of Dentistry, University of the Republic, Uruguay.

Conflict of interest
The authors declare that they have no conflict of interest.

Ethics
This study was approved by the Human Research Ethics Committee, number 091900-000351-19, School of Dentistry, Universidad de la República, Uruguay.

Authors contributions
Natalia Tancredi-Cueto: conception and design of the study; Data acquisition; analysis of data; discussion of the results; drafting of the manuscript; approval of the final version of the manuscript. Gabriela Vigil-Bastitta: data acquisition; IHC processing; approval of the final version of the manuscript. Ronell Bologna-Molina: conception and design of the study; drafting of the manuscript; approval of the final version of the manuscript. Verónica Beovide-Cortegoso: conception and design of the study; discussion of the results, drafting of the manuscript; approval of the final version of the manuscript.