MCM3: A Novel Proliferation Marker in Oral Squamous Cell Carcinoma

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Abstract: The present study sought to evaluate and compare the immunoexpression of proteins minichromosome maintenance (MCM) 3 and Ki-67 in oral squamous cell carcinoma (OSCC) to assess the potential of these proteins as markers of cellular proliferation. Twenty-eight cases of OSCC, 9 of tumor-free resection margins (TM), and 4 of non-neoplastic oral mucosa (NNM) were subjected to immunohistochemistry to detect the expression of proteins MCM3 and Ki-67. All OSCCs demonstrated positivity for both proteins. In these tumors, greater MCM3 immunoreactivity was observed in comparison with Ki-67, whereas TMs and NNMs exhibited greater Ki-67 expression compared with MCM3. The immunoexpression of Ki-67 seemed to be influenced by the inflammatory process, particularly in TM and NNM. Our findings indicate that although both MCM3 and Ki-67 represent reliable markers of cellular proliferation in OSCC, as MCM3 expression does not appear to be influenced by external factors, this protein may emerge as a novel marker of cellular proliferation in these types of tumors.

Key Words: squamous cell carcinoma, cellular proliferation, immunohistochemistry

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Oral cancer, despite being readily preventable and detectable at early stages, continues to be a public health concern worldwide.[1,2] The International Agency for Research on Cancer estimates that approximately 320,000 new cases and 157,000 deaths will occur in 2015 alone, 80% afflicting developing countries. In Brazil, 15,290 new cases of oral cavity cancer were predicted in 2015, with oral squamous cell carcinoma (OSCC) being the predominant histologic type, as over 95% of all cases occur at this anatomic site.[1,3,4] When diagnosed at later stages, oral cancer carries a high morbidity and mortality, and $< 50\%$ of all patients are expected to survive longer than 5 years.[1,5]

The proliferative capacity of neoplastic cells, an important feature in tumor growth,[6] is considered as an important prognostic indicator.[7] Minichromosome maintenance (MCM) proteins have recently come under investigation, as these helicases play a fundamental role in the replication of eukaryotic DNA by ensuring that this process occurs only once during each cell cycle.[7,8] The MCM3 protein, similar to other polypeptides comprising the MCM complex, is present at lower intracellular levels in differentiated or quiescent cells.[9]

Classic markers of cellular proliferation, such as protein Ki-67, have been used widely in the assessment of a variety of human tumors, including sarcomas, lymphomas, and breast and prostate neoplasia.[10] Lee et al[7] reported that MCM3 protein expression provides more sensitive and reliable results with which to evaluate the proliferative profile of cell populations, as this protein continues to be expressed for longer than Ki-67. Furthermore, Lameira et al[8] emphasized that the protein expression of Ki-67 offers imprecise information with respect to malignant neoplasia, as it incorporates the total fraction of cells within the cell cycle, regardless of whether these cells will eventually undergo differentiation in the absence of any relation to a malignant phenotype. In addition, Ki-67 expression may also occur when DNA synthesis is blocked or in apoptotic cells.[8,11]

The use of MCM proteins as markers of cellular proliferation has been described in some human tumors, such as salivary gland tumors,[12] thyroid papillary carcinoma,[7] and melanoma.[13] In OSCC, MCM2 protein immunoreactivity is considered as a better marker of tumor prognosis[14–16] than Ki-67.[11] Nevertheless, to the best of our knowledge, few studies have attempted to assess the role of the protein MCM3 in oral cancer.[8,10] The present study therefore aimed to evaluate and compare the

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immunoexpression of MCM3 and Ki-67 to assess the potential of these proteins as markers of cellular proliferation in OSCC.

MATERIALS AND METHODS

The present research proposal received approval from our host institution's review board. The expression of proteins MCM3 and Ki-67 was investigated in 28 cases of OSCC. All tumors were clinically classified by 2 experienced pathologists according to the TNM Classification of Malignant Tumours (2002), in addition to being histologically classified in accordance with the WHO criteria (2005). Nine cases of tumor-free resection margins (TM) were included, with oral epithelial dysplasia (OED) present in 5 (55.55%) cases: 3 (60%) of these were classified as mild, 1 (20%) was moderate, and 1 (20%) as severe. For comparison purposes, the expression of these 2 proteins was also quantified in 4 histologically normal non-neoplastic oral mucosa (NNM) tissue samples obtained from the removal sites of unerupted mandibular third molars in healthy patients—that is, nonsmokers who refrained from alcohol use. Table 1 lists the clinical and histologic characteristics of the OSCC cases studied.

The presence of inflammation was also observed in all OSCC, TM, and NNM samples in accordance with the amount of inflammatory cells dispersed throughout the subjacent connective tissue.

TABLE 1. Clinical and Histologic Characteristics of Patients With Oral Squamous Cell Carcinoma

Clinical Parameters	Total [n (%)]
Sex	
Male	20 (71.42)
Female	8 (28.58)
Tumor size	
T1-T2	14 (50)
T3-T4	14 (50)
Metastasis—lymph node	
N0	7 (35)
N1-N3	21 (75)
Metastasis—distance	
M0	23 (82.14)
Mx	5 (17.86)
Clinical status	
I-II	3 (10.72)
III-IV	25 (89.28)
Muscular invasion	
Yes	13 (46.42)
No	15 (53.58)
Bone invasion	
Yes	4 (14.28)
No	24 (85.72)
Perineural/vascular invasion	
Yes	2 (7.14)
No	26 (92.86)
Histologic gradation	
Well-differentiated	19 (67.85)
Moderately differentiated	8 (28.58)
Poorly differentiated	1 (3.57)
Anatomic site	
Tongue	15 (53.57)
Floor of mouth	8 (28.57)
Retromolar region	3 (10.71)
Gingiva	2 (7.15)

Immunohistochemistry

Sections (4-μm thick) were obtained from formalin-fixed paraffin-embedded specimens. Histologic sections were deparaffinized in xylene, then rehydrated with alcohol, and subsequently subjected to antigen retrieval (MCM3, citrate pH 9.0; Ki-67, citrate pH 6.0) under moist heat for 45 minutes to reveal antigenic epitopes. Endogenous peroxidase activity was subsequently blocked (Peroxidase Blocking Solution; Dako, Carpinteria) for 10 minutes under dark conditions, and tissue protein blocking was also performed for 10 minutes (Protein Blocking Solution; Dako). The primary antibodies (MCM3, Clone 101; Dako; Ki-67, Polyclonal; Abcam) were incubated for 18 hours at 4°C.

After primary antibody incubation, horseradish peroxidase Link and horseradish peroxidase Enzyme reagents (Advance; Dako Corporation) were applied to tissue sections for 20 minutes each. Reactions were developed using 3,3-diaminobenzidine (Dako) for 5 minutes in a dark chamber, and all slides were subsequently counterstained with Harris' hematoxylin for 1 minute, and then mounted in natural Canada balsam. For negative controls, each primary antibody was substituted with normal serum of the same isotype.

Immunohistochemical Analysis

An Aperio digital microscope (Leica Microsystems, Wetzlar, Germany) was used to scan all slides, which were then displayed on an LCD monitor by the Aperio Image Scope software (Leica Microsystems). Five coincident and representative areas (×200 final amplification) of each OSCC case were selected, with each area containing no <75% of the tumor parenchyma. The same procedures were likewise carried out with respect to all TM and NNM samples.

Analysis of MCM3 and Ki-67 Proteins

The nuclear location of each antigen was noted, taking into account the cell type and whether staining occurred in the parenchyma and/or the stroma of OSCC cases, or in the epithelium and/or the lamina propria of TMs and NNMs. In the parenchyma/epithelium, positive and negative cells were first counted in each microscopic field; the number of positive cells was then divided by the total number of cells and semiquantification criteria, as described previously by Gurgel et al,[17] were applied to obtain scores as follows: negative (−), <5% of positively immunostained cells; 1+, 5% to 25%; 2+, 26% to 50%; 3+, 51% to 75%; 4+, >75% of positively immunostained cells.

Statistical Analysis

All data were subjected to a statistical analysis, and group comparisons were made under nonparametric testing (Mann-Whitney, Kruskal-Wallis) by the GraphPad Prism Software (GraphPad Software, San Diego). *P*-values corresponding to α—that is, ≤5%, were considered significant.

RESULTS

Low levels of MCM3 and Ki-67 protein expression were observed in well-differentiated tumors, whereas OSCCs that were smaller in size—that is, classified as clinical stages I or II with no evidence of metastatic lymph nodes and no muscle or bone invasion, showed greater immunoexpression for MCM3 (Table 2). In contrast, a higher positivity for the Ki-67 protein was seen in tumors with more aggressive parameters (T3 to T4, metastatic lymph nodes, clinical stages III to IV). Table 2 summarizes the immunoreactivity for MCM3 and Ki-67 with respect to the clinical and histologic parameters pertaining to OSCC.

A Higher MCM3 Expression was Observed in OSCC Compared With TM and NNM

All cases of OSCC exhibited positivity for the MCM3 protein, which was localized in the nuclei of parenchymal tumor cells. Immunostaining for this protein was predominantly scored as 4+ (n = 19; 67.85%), followed by 3+ (n = 6; 21.42%), 2+ (n = 2, 7.14%), and 1+ (n = 1, 3.59%) (Fig. 1). All TMs also demonstrated positivity for MCM3, with staining observed in cells within the epithelial compartment, mostly with a corresponding score of 2+ (n = 7; 77.77%), followed by 1+ (n = 2; 22.23%) (Fig. 1). Among the TM cases with OED (n = 5), all were scored as 2+. With respect to NNM, staining for MCM3 was more discrete and restricted to the basal layer of the epithelium, with scores of 1+ (n = 2; 50%), 2+ (n = 1, 25%), and 0 (n = 1, 25%) (Fig. 1).

TABLE 2. Levels of MCM3 and Ki-67 Protein Immunoexpression in Accordance With the Clinical and Histologic Parameters of OSCC

		MCM3		Ki-67	
	Total	Median	*P*	Median	*P*
Size					
T1-T2	14	1885	0.26	1246	0.36
T3-T4	14	1550		1636	
Lymph node					
N0	7	1976	0.14	1505	0.27
N1-N3	21	1586		1559	
Clinical stage					
I-II	3	2496	**0.04**	731	0.32
III-IV	25	1586		1528	
Muscular invasion					
Yes	13	1889	0.42	1743	0.19
No	15	1586		1242	
Bone invasion					
Yes	4	1228	0.12	1087	0.33
No	24	1866		1517	
Histologic grading					
Well-differentiated	19	1514	0.058	1242	0.07
Moderately differentiated	8	2546		2266	
Anatomic site					
Tongue	15	1586	0.26	1242	0.46
Mouth floor	8	2238		1854	
Retromolar region	3	1926		1505	

Value in bold are statistically significant ($P < 0.05$).
OSCC indicates oral squamous cell carcinoma; MCM3, minichromosome maintenance 3.

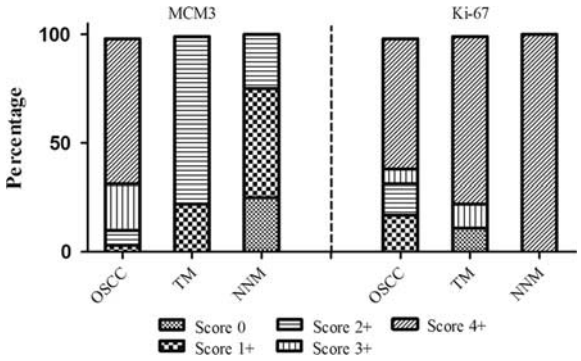

FIGURE 1. Expression distribution of MCM3 and Ki-67 in OSCC, TM, and NNM. MCM3 indicates minichromosome maintenance 3; NNM, non-neoplastic oral mucosa; OSCC, oral squamous cell carcinoma; TM, tumor-free resection margins.

Higher MCM3 protein expression was seen in OSCC tumors compared with TMs and NNMs ($P = 0.001$; Fig. 2B). Figure 3 illustrates the immunoreactivity for MCM3 in NNM (Figs. 3A, C), TM (Figs. 3E, G), and OSCC (Figs. 3I, K).

Higher Ki-67 Protein Expression in TM and NNM Compared With OSCC

All samples of OSCC and NNM, in addition to most TMs, demonstrated nuclear immunostaining for protein Ki-67. In OSCC, this expression was seen in both parenchymal and stromal tumor cells. In the first compartment, 4+ was the predominant score (n = 17; 60.71%), followed by 1+ (n = 5; 17.85%), 2+ (n = 4, 14.28%), and 3+ (n = 2; 7.16%) (Fig. 1). Regarding TMs, 8 cases (88.88%) were positive for Ki-67, with staining observed in epithelial cells and the lamina propria. In the epithelium, a score of 4+ predominated (n = 7; 77.77%), followed 3+ (n = 1, 11.11%) and 0 (n = 1, 11.11%) (Fig. 1). Among the cases of TM with OED (n = 5), all were scored 4+. Immunostaining for Ki-67 was scored as 4+ in all NNM cases (Fig. 1), with positive cells distributed throughout all layers of the epithelium. OSCC tumors exhibited lower Ki-67 expression in comparison with TMs and NNMs ($P = 0.03$; Fig. 2A). Figure 3 illustrates the immunoreactivity for Ki-67 in NNM (Figs. 3B, D), TM (Figs. 3F, H), and OSCC (Figs. 3J, L).

Proteins MCM3 and Ki-67 Behave Similarly in OSCC, but Differently in TM and NNM

In OSCC, increased immunoexpression of protein MCM3 was seen in comparison with Ki-67 (Fig. 4). In addition, cells positive for MCM3 were present exclusively in the tumoral parenchyma (Figs. 3I, K), whereas Ki-67 was positively stained in both the parenchymal and the stromal compartments (Figs. 3J, L). Nonetheless, significantly increased immunostaining for Ki-67 was observed in TMs and NNMs compared with

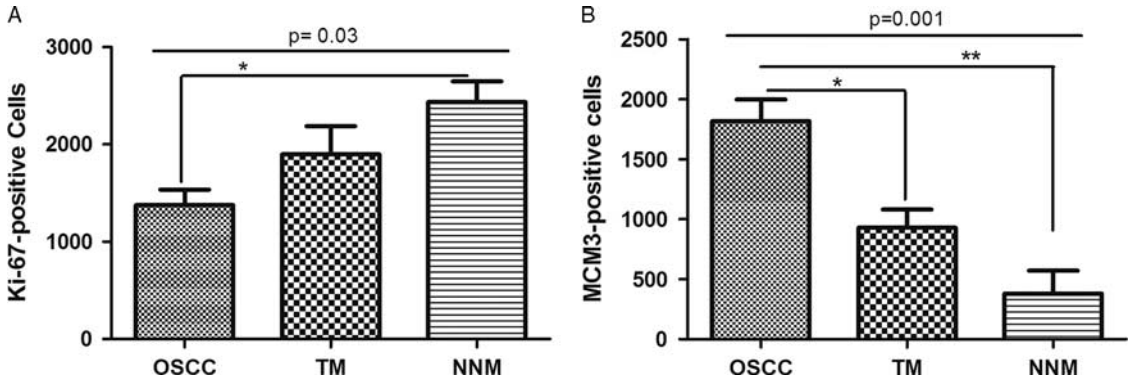

FIGURE 2. Distribution of Ki-67 protein expression (A) and MCM3 protein expression (B) in cases of OSCC, TM, and NNM. MCM3 indicates minichromosome maintenance 3; NNM, non-neoplastic oral mucosa; OSCC, oral squamous cell carcinoma; TM, tumor-free resection margins.

MCM3 ($P = 0.003$ and 0.01, respectively; Fig. 4). Notably, Ki-67 expression was seen in the epithelium and the lamina propria, whereas MCM3 was localized only in the epithelial compartment (Fig. 3A–H). In addition, in NNM, cells positive for MCM3 were identified in the basal layer of the epithelium, whereas Ki-67-positive cells were distributed throughout all epithelial layers (Fig. 3B).

DISCUSSION

The present study attempted to assess and compare the expression of 2 proteins associated with cellular proliferation, MCM3 and Ki-67, in OSCC, TM, and NNM. In OSCC samples, greater immunostaining for MCM3 was observed in comparison with Ki-67. In contrast, TMs and NNMs had higher Ki-67 expression in comparison with MCM3. Interestingly, particularly in TMs and NNMs, the immunoreactivity for Ki-67 seemed to be influenced by inflammation, as evidenced by the greater proliferative activity in epithelial cells.

The Ki-67 protein is one of the most studied markers of cellular proliferation. Its expression is initiated in the S phase,[17,18] increases throughout the G2 phase progressively, peaks in G1 phase,[18] and then disappears rapidly when cells reach G0.[9] The fraction of Ki-67-positive cells is frequently associated with the clinical course of tumors, such as cancers of the breast[19] and the lung.[20] Studies have shown that tumors with elevated Ki-67 expression are associated with an increased risk of recurrence and lower survival rates.[21–23] Relatedly, in OSCC, Ki-67 has been described as an important predictive marker of recurrence[24] and survival.[25]

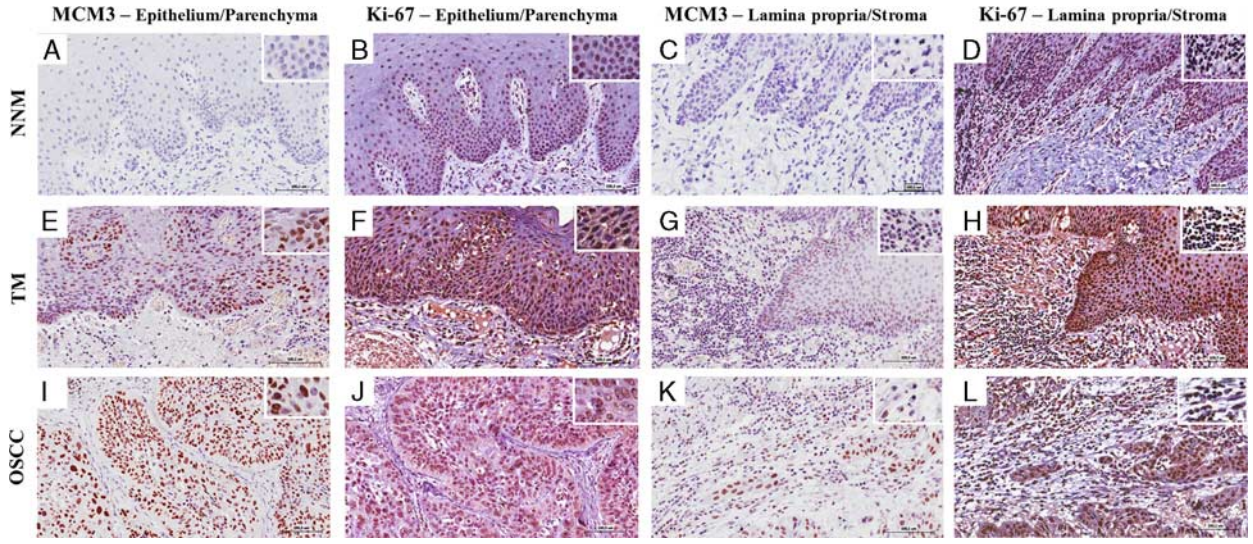

FIGURE 3. Immunostaining for minichromosome maintenance (MCM) 3 and Ki-67 in cases of non-neoplastic oral mucosa (NNM), tumor margin (TM), and oral squamous cell carcinoma (OSCC). MCM3: A and C, negatively scored NNM. E and G, TM exhibiting nuclear staining localized only in the epithelial compartment. I and K, OSCC exhibiting cells positive for MCM3 exclusively in the tumoral parenchyma. Ki-67: B, Positively scored epithelial cells distributed throughout all epithelial layers in NNM. D, Positively scored inflammatory cells distributed in the lamina propria of NNM. F and H, Ki-67 expression in the epithelium and the lamina propria of TM. J and L, positive cells in both the parenchymal and the stromal compartments.

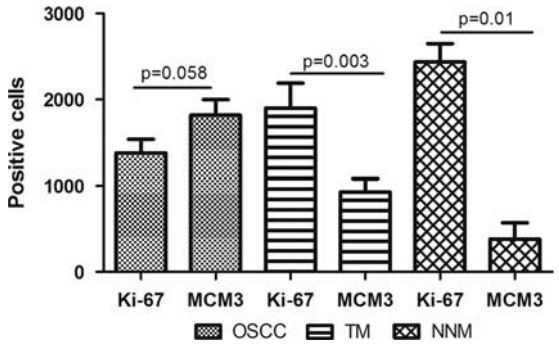

FIGURE 4. Comparison of Ki-67 and MCM3 protein expression between OSCC, TM, and NNM. MCM3 indicates minichromosome maintenance 3; NNM, non-neoplastic oral mucosa; OSCC, oral squamous cell carcinoma; TM, tumor-free resection margins.

Unfortunately, it has not yet been possible to establish a threshold level for Ki-67 detection that would allow for the differentiation of tumors with varying prognoses.[26,27] In breast cancer, for example, a previous study suggested a level of 25% for Ki-67-positive cells, although prospective validation studies have not yet been conducted.[27]

The 6 members of the MCM protein family (MCM2-7) comprise a group of DNA-binding proteins that play an important role in the initiation and regulation of DNA replication and cell cycle progression.[7,12,28] Notably, the expression of MCM3, a helicase fundamental to eukaryotic DNA replication,[7,8,15] is maintained for longer than that of Ki-67,[7] continuing until the transition between phases G0 and G1.[15]

Simon and Schwacha[29] concluded that because the MCM protein complex plays a role in cellular proliferation, any alterations that lead to an increased activity by this helicase are thus associated with cancer development. To this end, studies have indeed reported that increased MCM3 expression is associated with a worse prognosis in gliomas,[30] salivary gland tumors,[12] thyroid carcinoma,[7] and melanoma.[13] In OSCC and premalignant oral lesions, MCM3 is regarded as a prognostic and diagnostic marker[10] that some consider to be superior to Ki-67.[8]

One of our findings was that less aggressive OSCCs of smaller size, without metastatic lymph nodes or any muscle or bone invasion and classified as clinical stages I or II, exhibited more marked immunostaining for MCM3 and Ki-67. This evidence must be interpreted in the context of the tumor biology, because in early stages, the proliferative tumor compartment is predominantly responsible for the death of malignant cells, whereas at later stages, neoplastic cells acquire an invasive capability and promote tumor progression.[6] Furthermore, more differentiated tumors exhibit lower MCM3 and Ki-67 immunoreactivity, which is generally associated with an improved prognosis,[14] as these cells still retain some differentiation potential.

Particularly in comparison with NNM, OSCCs exhibit higher MCM3 protein expression, indicating that cellular proliferation is a key event in tumor growth and progression.[6,7] In the NNM samples considered herein, MCM3-positive cells were seen only in the basal layer of the epithelium, whereas the Ki-67 protein was found to be distributed throughout all layers of the epithelium. Moreover, Ki-67 expression was higher in NNMs than in OSCCs. Considering that inflammation of the oral mucosa is a frequent occurrence, taken together with the presence of Ki-67-positive cells dispersed throughout the connective tissue (lamina propria/stroma), it is logical to conclude that the inflammatory process seems to influence the expression of Ki-67,[31–33] but not MCM3. Within this context, Ki-67 expression has been previously associated with the secretion of proinflammatory factors, such as NF-κB in chronic inflammatory dermatosis—for example, psoriasis vulgaris.[34] Furthermore, some studies have demonstrated that the secretion of cytokines and growth factors during inflammatory events may consequently modulate cellular proliferation, growth, and differentiation.[31–33] Relatedly, a previous report found a mitogenic effect resulting from PDGF growth factor expression in keratinocyte cell cultures, with consequently increased levels of Ki-67.[35]

Premalignant lesions of the mouth, which often precede OSCC, are generally difficult to differentiate from benign alterations. As such, the use of biomarkers of cellular proliferation can aid in prognosis establishment, as greater numbers of cells undergoing division increases the chances of genomic instability, thereby promoting the emergence of aggressive clones.[36] Accordingly, some studies attempting to evaluate the use of biomarkers capable of detecting early-stage oral dysplasia[16,37] propound the superiority of MCM family proteins over Ki-67. Corroborating these reports, our results demonstrate a more reliable pattern of immunostaining for MCM3 in TMs, despite the higher levels of Ki-67 protein immunoreactivity detected. Nevertheless, we must stress that the expression of this protein indeed appears to be influenced by the inflammatory process in both NNMs and TMs.

It is clear that biomarkers of cellular proliferation can aid in the interpretation of morphologic alterations occurring during neoplastic initiation and transformation processes. The analysis of proliferative tumor activity, in association with clinical and histopathological data, provides useful information regarding the biological and prognostic behavior of neoplasia—for example, by providing an estimation of the rate of tumor growth as well as assisting in treatment planning.[29] Despite widespread investigation on the use of biomarkers to improve the assessment of oral cancer prognosis, the standardized clinical application of prognostic markers remains a goal yet to be achieved.[15]

In conclusion, the search for alternative markers of cellular proliferation must continue, particularly in light of the potential they offer to significantly enhance the clinical management of patients affected by malignant neoplasia, as well as in the hope that these will eventually lead to reduced morbidity and mortality not only in epithelial

dysplasia, but also in other forms of cancer. Our findings suggest that although both MCM3 and Ki-67 proteins represent proven markers of cell proliferation in oral cancer, the expression of MCM3 does not appear to be influenced by external factors—for example, inflammation, which seems to be the case when quantifying Ki-67 immunoexpression. Nevertheless, further studies must be conducted to assess the influence (or a lack thereof) that proteins MCM3 and Ki-67 may have on the inflammatory process. It is our hope that the present results regarding MCM3 immunoreactivity in OSCCs and TMs may serve to further the emergence of MCM3 as a novel marker of cellular proliferation in these types of tumors.

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