Abstract

Proliferative activity and differentiation of tumor's cells is an essential parameter that determine the course and the prognosis of the disease. The aim of this study was to investigate the immunoexpression patterns for Ki-67, MCM3 and p27 in oral squamous cell carcinoma (OSCC) as well as to address the correlation with patient's survival and clinicopathological features. Fifty-one patients were enrolled. The clinical (tumor size, tumor staging, lymph nodal involvement and survival) and pathological characteristics (differentiation grade) of the patients were recorded. The expression of Ki-67, MCM3 and p27 was evaluated with immunohistochemical methods using paraffin blocks. The mean-age of patients was 63.89 year. The association was examined for statistical significance using chi-square test. Overall survival rates were estimated by the Kaplan-Meier method and compared using a log rank test ($P > 0.05$). The results indicated a statistically immunostaining significance for the expression of Ki-67 and survival rate ($p = 0{,}00882$). From these results, the present study suggest that high Ki-67 expression found in OSCC patients may contribute to tumor growth and survival rate.

Key Words: Squamous Cell Carcinoma, Biomarkers, Survival.

Introduction:

Oral Squamous Cell Carcinoma (OSCC) represents 95% of all malign neoplasms that occur in the oral cavity [1,2]. It is an aggressive neoplasm with unpredictable biological behavior and unfavorable prognosis [3]. Actually, the decisions about therapeutic modalities used in OSCC are based on clinical features, including the tumor size (T), involvement of lymph nodes (N) and presence of distant metastasis (M), named TNM staging system [4, 43]. Although useful, these criteria do not explain why lesions diagnosed in early stage present a poor prognosis. In this sense, the identification of molecular markers associated to the tumor staging, lymph nodal metastasis, as well as the prognosis, may be useful tool to identify the lesion's aggressiveness, especially those one in initial stage, as soon as in registering efficient chemotherapy that acts in the signalizing via that these proteins act [5]. Ki-67 is expressed in proliferative cells, but quickly disappears when the cell enters in a rest state. This characteristic has stimulated the use of Ki-67 to demonstrate a fraction of cells that is proliferating at a given malign neoplasm [6, 7]. The protein p27 is a suppressor tumor and mediates the formation CDK2-Cyclin, inhibiting the entrance on S cellular cycle stage [8, 9]. Other protein group that has been recently investigated is the Mini-chromosome Maintenance Proteins (MCM). These proteins are evolved in the early stages of the eukaryotic genome replication and is believed to serve as normal replication machinery component [10]. All the six members of MCM family, MCM2 to MCM7, form an important complex and its regulation is essential to DNA replication [11, 12]. It has been recently demonstrated that the entry in the quiescent state by the cell causes a rapid disappearance of the Ki-67 protein, subsequently followed by the disappearance of MCM3 expression and an increase in p27 protein expression [13]. This information indicates that the assessment of these proteins can be a useful tool to establish which

cells are proliferating, at resting stage or differentiating. The aim of this study is to evaluate the association of two proliferation markers – ki-67 and MCM3 – and one of differentiation – p27 – associated with clinical (age, gender, smoking, alcoholism, primary tumor site, tumor size, lymph node metastasis and disease stage), histological (high-differentiated, moderately-differentiated and poorly-differentiated) and survival criteria.

Materials and methods

Specimens and inclusion criteria

A total of 51 paraffin-embedded biopsy specimens of OSCC from 33 (64.7%) males and 18 (35.3%) females for Ki-67; 27 (52.95%) males and 24 (47.05%) females for MCM3; 34 (66.66%) males and 17 (33.33%) females for p27 were selected in the period between 1996 and 2014 from the Service of Oral Pathology of the University Hospital João de Barros Barreto (Pará, Brazil). Samples were selected from patients (with a diagnosis confirmed by histopathology) who had primary tumours of the oral cavity with surgery as the only treatment modality. For analysis of the histologic grade were used 79 samples of tissue squamous cell carcinoma. The required data were obtained from patient records, summarized on standardized forms and stored in a database. The ethical committee of the University Hospital João de Barros Barreto approved this work under approval number 51641/12.

Immunohistochemistry

Three micrometer mounted sections fixed in 4% formalin were dewaxed with xylene and dehydrated in different ethanol gradients. The slides were immersed in 10 mM EDTA (pH 8.0) for 15 min in a microwave oven. Peroxidase activity was blocked with

6% hydrogen peroxide and methanol solution in two baths (15 min each) at room temperature. Endogenous peroxidase quenching was performed by a 6% hydrogen peroxide and methanol solution (v/v) in two baths (15 min each). After washing in Tris buffer (pH 7.4), slides were incubated with the primary antibodies for anti-ki67 (1:150, Dako, Clone MIB-1, Carpinteria, CA, USA), MCM3 (1:100, Dako, Clone 101, Carpinteria, CA, USA), and anti-p27 (1:200, Dako, clone SX53G8, Carpinteria, CA, USA) for 18h at 4°C. The slides were subsequently exposed to the avidin–biotin complex (LSAB-Kit + HRP; Dako Cytomation) and to the 3,3′-diaminobenzidine chromogen (DAB+; Dako Cytomation).

Sections were counterstained with Mayer's haematoxylin, dehydrated in ethanol, cleared in xylene and mounted. Breast cancer tissues were used as positive control for all antibodies. The negative control was obtained by omitting the primary specific antibody during the reaction. The reaction results were considered positive when brown-stained cells were observed, characterizing the presence of DAB in the immunohistochemistry reaction. The sections that underwent immunohistochemical reactions were analyzed by a quantitative system of points to reduce possible distortions related to the heterogeneity of samples. The scoring system was previously published in the literature [14]. The analysis was based on intensity and distribution of staining. The distribution of stained cells was analysed as follows: 0 (0%), 1 (1% to 50%), and 2 (51% to 100%). The intensity of staining was rated as follows: 0 (no staining), 1 (mild staining), 2 (moderate staining), and 3 (strong staining). The immunostaining for all used antibodies was considered positive when cells from any tumor mass layer were observed. In addition, the immunolabelling was considered specific when the immunoreactivity was mostly restricted to the nuclear region. Two independent pathologists blind to the experimental groups evaluated the immunolabelled sections. In

the event of disagreement, the two pathologists observed the sections to achieve a consensus.

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences software for Windows, version 18.0 (SPSS Inc, Chicago, IL, USA). Associations between Ki-67, MCM3 and p27 expression and clinicopathological parameters were examined for statistical significance using a chi-square test. Overall survival rates were estimated by the Kaplan–Meier method and compared using a log rank test. A P value of <0.05 was considered significant.

Evaluation of histological grade and clinicopathological features

The histological assessment followed the parameters of the World Health Organization [44] and was carried out by two pathologists without prior knowledge of the clinical data of the patients. The relationships between ki67, MCM3 and p27 protein and clinicopathological features, including age, gender, tabagism, alcohol intake, primary tumor site, tumor size, lymph node metastasis, disease stage were assessed in 51 samples and histological grade were assessed in 79 samples.

Results

The required data were obtained from patient records, summarized on standardized forms and stored in a database. The mean patient age was 63.89 (range, 36-95) years. The mean follow-up of the patients was 39.5 months (range, 36-43). In our study, there were no cases with distant metastasis. The patients' clinicopathological features are summarized in table 1 for Ki-67, table 2 for MCM3 and table 3 for p27. The chi-square test showed no statistical difference between Ki-67, MCM3 and p27 immunolabelling and other clinicopathological features, as age, gender, smoking, alcoholism, primary

tumor site, tumor size, lymph node metastasis, disease stage and histological grading. The Kaplain-Meier test analyzed the general survival for Ki-67, MCM3 and p27.

Ki-67 expression is related with overall survival

Immunohistochemical staining for Ki67 is widely used as a surrogate for proliferation in tumor samples, being an important tool to analyze its association with prognosis. The results showed positive immunostaining for Ki-67 in 40/51 (78,43%) (Fig.1a) and negative immunolabelling for Ki-67 in 11/51 (21.57%) samples. Patients that expressed Ki-67, later one month of follow-up have 96% of probability of survival and after 36 months of follow-up is 26,83% (Fig.2a). The overall survival for Ki-67 indicates that a patient with positive expression is lower than a patient with negative (log-rank test, p = 0,00882) (Fig.3). It was confirmed by hazard Cox that a patient with positivity for Ki-67 has five times more risk of death than a patient with negative Ki-67 immunoreactivity. The relationships between ki67 and clinicopathological features demonstrated any significance, including age, gender, smoking, alcoholism, primary tumor site and histological grade.

MCM3 is not related with overall survival or clinicopathological features

Recent studies have proposed that MCM7 may be sensitive proliferative and prognostic markers of various malignant tumors. Our results showed positive immunostaining for MCM3 in 44/51 (86,27%) (Fig. 1b) and negative immunolabelling for MCM3 in 7/51 (13,73%) samples. For MCM3, the general survival rate later one month of follow-up is 92,10%, but presented 12,30% later 43 months of follow-up (Fig. 2b). The relationships between MCM3 and clinicopathological features, including age, gender, smoking, alcoholism, primary tumor site and histological grade had no significant data.

P27 is not related with overall survival or clinicopathological features

Studies to evaluate expression of cell-cycle regulatory proteins p27 have been showing diagnostic or prognostic value. We have had positivity for p27 in 39/51 (76,47%) (Fig.1c) and negative immunolabelling for p27 in 11/51 (21.57%) samples. In addition, p27 showed later one month of follow-up is 90,09%, and later 36 months of follow up is 24,50% (Fig. 2c). The relationships between p27 and clinicopathological features, including age, gender, smoking, alcoholism, primary tumor site and histological grade presented no significance on the analysis.

The tumor size, lymph nodal involvement and tumor staging are related with survival rates

Clinicopathological characteristic as tumor size, lymph node involvement and tumor staging have been related as symptoms of poor prognosis for OSCC. In our study, the Kaplain-Meier curve indicates that the survival rate of a patient with OSCC size T3 or T4 is significantly lower than a patient with size T1 or T2 (log-rank test, samples Ki67 p= 0.000029; samples MCM3 p= 0.0063; samples P27 p= 0.00055) (Fig.4). In samples of p27, the Kaplain-Meier curve indicates that the survival rate of a patient with OSCC lymph nodal disease N2 or N3 is significantly lower than a patient with N0 or N1 (p = 0,0429) (Fig. 5). The survival rate for a patient with disease staging III or IV is lower than a patient with staging I or II (log-rank test, samples Ki67 p= 0.00101; samples MCM3 p= 0.00831; samples P27 p= 0.000209) (Fig. 6).

Discussion

OSCC is the most frequent cancer that presents lymph nodes involvement and distant metastatic potential [15]. Although a better understand of cancer progression have been made in the last few years, the prognosis of OSCC is still presented with less than 50% of patients free of disease in 5 years [16, 40]. Based on it, the immunohistochemical

reaction represents a useful technique to evaluate the aggressiveness of several human malignancies when associated to clinical and histopathological aspects [17].

In this study we explored the immunoreactivity pattern for three important markers of proliferations and differentiation (Ki-67, MCM3 and p27) in OSCC samples and their relation with clinicopathological features and survival rates. The overall survival for positive Ki-67 expression is lower than a patient with negative immunoreactivity. Nonetheless, tumor size T3 and T4 decreases the survival when related to T1 and T2, as well as, the survival rate of patients with lymph node metastasis N2 and N3 is significantly lower than in patients with N0 or N1. Additionally, the survival rate for a patient with disease staging III or IV is lower than a patient with staging I or II.

In our previous study [18], we investigated the expression of Ki-67, MCM3 and p27 in oral leukoplakia and OSCC samples, which was noted that mutual antagonism between the circuits that control cell proliferation and cell differentiation, once the expression of p27 was lower in cases ki67 and MCM3 were high. When they related the immunostaining of p27 and MCM3 in all samples, they observed a strong correlation, which did not happen with the correlation between p27 and Ki-67. They related that this difference may be attributed to the fact that MCM3, besides being expressed in proliferating epithelial cells, perhaps, immunostained cells that were ready to enter the cell cycle. Based on it, we started to explore the relation of these proteins in OSCC associated to clinocopathological features and survival rates.

We found that the relation of Ki-67 expression indicates that the patient tended to have an overall survival lower than a patient with negative immunostaining, presenting 5 times more risk of death than a patient with negative Ki-67 immunoreactivity, while the expression for MCM3 showed no significant data. Some studies have commonly recognized that proliferation-associated antigen Ki67 was one of the best known

predictors for survival in patients with malignant diseases [19, 20], such as in lung cancer [21], breast cancer [22] and prostate cancer [23]. Because of its high sensitivity and marking specificity to cell proliferation in neoplastic tissues, it has been used to evaluate the neoplasm's aggressiveness [16].

Actually, one of the most innovative proteins used as proliferative markers is the MCM family (MCM2 to MCM7), more specifically MCM3. Some studies have evaluated the expression of the protein and its relation with poor prognosis in malignant melanoma [41], astrocytoma [24] and medulloblastoma [25]. All the six types of this family protein are plentiful in the cellular cycle, but its expression rapidly decline during the quiescence and cellular differentiation, which makes them specific proteins that may be used in the cellular cycle. The antibodies MCM proteins detect more proliferative cell than other proliferation markers, such as Ki-67 and the Proliferating Cell Nuclear Antigen (PCNA) [26]. Although it has been related that MCM3 is a better proliferative marker than Ki-67 [18, 42], our results showed none relation between MCM3 immunoreactivity and clinicopathological rates or survival. To the best of our knowledge, it has never been described in the literature a study that verify the survival degree and immunostaining related to MCM3 in OSCC.

P27 is a cell cycle regulator that was identified as a CDK inhibitor of the cyclin E/CDK2 complex [27]. It mediates cell cycle arrest by impairing the ability of cyclin E to promote G1-S transition. This function is regulated primarily by the amount of nuclear p27 [28]. P27 degradation via ubiquitination causes cyclin E activity and cell cycle progression [9]. Deletions, methylations and protolithic aberrations may generate a lower P27 expression [29].

p27 expression has been described as an important predictor associated with poor prognosis of patients [27, 30], such as in laryngeal squamous cell carcinomas [31],

OSCC and oropharynx [32] . In our study we have not found any prognostic significance in OSCC associated with p27. Perisanidis et al. [33] assessed the association between clinicopathological variables including the survival of the patients and the expression of biomarkers and observed any relationship between clinicopathological features and survival, what is in accordance to our results. The only significant data was the relation of p27 expression with an increase of the risk of lymph nodal involvement [34, 35].

Additionally, we showed the clinicopathological features associated to patient's survival. The overall survival rate of a patient with OSCC size T3 or T4 is lower than a patient with size T1 or T2 [36, 37, 38]. Aditionally, the overall survival demonstrated that a patient with tumor staging III or IV is lower than a patient with staging I or II [39].

Thus, in our work, Ki-67 expression was related with a decrease in the survival rate. As well as, the poor prognosis was related with the tumor size. It was observed a decrease in the survival rate for samples of p27 and lymph node metastasis. In addition, for Ki-67, MCM3 and p27 showed a decrease of survival when tumor size and disease stage were analyzed. In conclusion, differentiation studied the major factor in the prognosis was index proliferation by Ki-67 expression and size tumor. We suggest studies with more than 5-years of follow up to evaluates the behavior of the proteins.

Acknowledgements

This work was supported by the Brazilian Agency FAPESPA – Fundação Amazônica Paraense de Amparo à Pesquisa - and fellowship from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Brazil.

Conflict of interest

The authors declare that they have no conflict of interest.

Figure Legends

Fig. 1 Immunostaining in oral squamous cell carcinoma. **a** samples of Ki67. **b** samples of MCM3. **c** samples of P27 (40x magnification).

Fig. 2 Kaplan-Meier curve showing the proportion of patients according to overall survival. **a** samples of Ki67. **b** samples of MCM3. **c** samples of P27.

Fig. 3 Kaplan-Meier curve showing difference between Ki67 expression -positive and -negative patients, p=0.0082.

Fig. 4 There was significant difference in survival associated and tumour size (T -status). **a** samples of Ki67 (p= 0.000029). **b** samples of MCM3 (p= 0.0063). **c** samples of P27 (p= 0.00055).

Fig. 5 There was significant difference in survival associated and lymph node metastasis (N –status) for samples of P27, p=0.0429.

Fig. 6 There was significant difference in survival associated and tumor stage. **a** samples of Ki67 (p= 0.00101). **b** samples of MCM3 (p= 0.00831). **c** samples of P27 (p= 0.000209).

Referências

1. Chin D, Boyle GM, Williams RM, et al. Novel markers for poor prognostic in head and neck cancer. Int J Cancer 2005; 113: 789–97.
2. Lo WL, Kao SY, Chi LY, et al. Outcomes of oral squamous cell carcinoma in Taiwan after surgical therapy: factors affecting survival. *J Oral Maxillofac Surg* 2003;61:751-58
3. Riveira C, Venegas B. Histological and a molecular aspects of oral squamous cell carcinoma (Review). Oncology letters, 2013;8: 7-11.

4. Denoix PF. Tumor, Node and Metastasis (TNM). Bull Inst Nat Hyg (Paris) 1944;1:1–69
5. Vora HH, Shah NG, Patel DD, Trivedi TI, Chikhlikar PR. Prognostic significance of biomarkers in squamous cell carcinoma of the tongue: multivariate analysis. J Surg Oncol. 2003 Jan;82(1):34-50.
6. Freudlsperger C. *et al.* Ki-67 expression predicts radiosensitivity in oral squamous cell carcinoma. Int J Oral Maxillofac Surg. 2012; 41:p. 965-69.
7. Klimowicz AC, Bose P, Nakoneshny SC, Dean M, Huang L, Chandarana S, Magliocco AM, Wayne Matthews T, Brockton NT, Dort JC. Basal Ki67 expression measured by digital image analysis is optimal for prognostication in oral squamous cell carcinoma. Eur J Cancer. 2012 Sep;48(14):2166-74.
8. Auerkari, EI. *et al.* Expression of p27Kip1 and E-cadherin in Head and Neck Squamous Cell Carcinoma of Indonesian Patients. Open Dent J. 2014;8:136-43.
9. Jameson MJ, *et al*. Activation of the insulin-like growth factor-1 receptor alters p27 regulation by the epithelial growth factor receptor in oral squamous carcinoma cells. J Oral Pathol Med. 2013 Apr;42(4):332-8.
10. Han X, Mayca Pozo F, Wisotsky JN, Wang B, Jacobberger JW, Zhang Y. Phosphorylation of Minichromosome Maintenance 3 (MCM3) by Checkpoint Kinase 1 (Chk1) Negatively Regulates DNA Replication and Checkpoint Activation. J Biol Chem. 2015 May;290(19):12370-8.
11. Szelachowska J, *et al.* Correlation of metallothionein expression with clinical progression of cancer in the oral cavity. Anticancer Res. 2009;29:589-96.
12. Tamura T, Shomori K, Haruki T, Nosaka K, Hamamoto Y, et al. Minichromosome maintenance-7 and geminin are reliable prognostic markers in patients with oral squamous cell carcinoma: immunohistochemical study. J Oral Pathol Med. 2010; 39:328–334.
13. Endl E. *et al.* The expression. Of ki-67, MCM 3, and p27 defines distinct subsets of proliferating, resting, and differentiated cells. J Patho. 2001;195:457-52.
14. Tsurutani J. *et al*. Tobacco components stimulate Aκt-dependent proliferation and NF-κB-dependent survival in lung cancer cells. Carcinogenesis. 2005;26:1182-95.
15. Zargaran M, Eshghyar N, Baghaei F, Moghimbeigi A. Assessment of cellular proliferation in oral verrucous carcinoma and well-differentiated oral squamous cell carcinoma using Ki67: a non-reliable factor for differential diagnosis? Asian Pac J Cancer Prev. 2012;13(11):5811-5.

16. Pontes FS, Carneiro JT Jr, Fonseca FP, da Silva TS, Pontes HA, Pinto Ddos S Jr. Squamous cell carcinoma of the tongue and floor of the mouth: analysis of survival rate and independent prognostic factors in the Amazon region. J Craniofac Surg. 2011 May;22(3):925-30.
17. Pontes HA, Pontes FS, de Jesus AS, Soares MC, Gonçalves FL, de Lucena Botelho T, do Carmo Ribeiro J, Dos Santos Pinto D Jr. p-Akt and its relationship with clinicopathological features and survival in oral squamous cell carcinoma: an immunohistochemical study. J Oral Pathol Med. 2015 Aug;44(7):532-7.
18. Lameira AG. *et al.* MCM3 could be a better marker than Ki-67 for evaluation of dysplastic oral lesions: an immunohistochemical study. J Oral Patho Med. 2014 Jul;43(6):427-34.
19. Iamaroon A, Khemaleelakul U, Pongsiriwet S, Pintong J. Co-expression of p53 and Ki67 and lack of EBV expression in oral squamous cell carcinoma. J Oral Pathol Med. 2004 Jan;33(1):30-6.
20. Montebugnoli L, Badiali G, Marchetti C, Cervellati F, Farnedi A, Foschini MP. Prognostic value of Ki67 from clinically and histologically 'normal' distant mucosa in patients surgically treatedfor oral squamous cell carcinoma: a prospective study. Int J Oral Maxillofac Surg. 2009 Nov;38(11):1165-72.
21. Martin B, Paesmans M, Mascaux C, Berghmans T, Lothaire P, Meert AP, Lafitte JJ, Sculier JP. Ki-67 expression and patients survival in lung cancer: systematic review of the literature with meta-analysis. Br J Cancer. 2004 Dec.;91(12):2018-25.
22. Yang XQ, Wang FB, Chen C, Peng CW, Zhang JF, Li Y. High Ki-67 expression is a poor prognostic indicator of 5-year recurrence free survival in patients with invasive breast cancer. Asian Pac J Cancer Prev. 2011;12(11):3101-5.
23. Aaltomaa S, Kärjä V, Lipponen P, Isotalo T, Kankkunen JP, Talja M, Mokka R. Expression of Ki-67, cyclin D1 and apoptosis markers correlated with survival in prostate cancer patients treated by radical prostatectomy. Anticancer Res. 2006 Nov-Dec;26(6C):4873-8.
24. Söling A, Sackewitz M, Volkmar M, Schaarschmidt D, Jacob R, Holzhausen HJ, Rainov NG. Minichromosome maintenance protein 3 elicits a cancer-restricted immune response in patients with brain malignancies and is a strong independent predictor of survival in patients with anaplastic astrocytoma. Clin Cancer Res. 2005 Jan 1;11(1):249-58.

25. Lau KM, Chan QK, Pang JC, Li KK, Yeung WW, Chung NY, Lui PC, Tam YS, Li HM, Zhou L, Wang Y, Mao Y, Ng HK. Minichromosome maintenance proteins 2, 3 and 7 in medulloblastoma: overexpression and involvement in regulation of cell migration and invasion. Oncogene. 2010 Oct 7;29(40):5475-89.
26. Scott IS, Odell E, Chatrath P, et al. A minimally invasive immunocytochemical approach to early detection of oral squamous cell carcinoma and dysplasia. Br J Cancer. 2006; 94: 1170–5.
27. Gao L, Huang S, Ren W, Zhao L, Li J, Zhi K, Zhang Y, Qi H, Huang C. Jun activation domain-binding protein 1 expression in oral squamous cell carcinomas inversely correlates with the cell cycle inhibitor p27. Med Oncol. 2012 Dec;29(4):2499-504.
28. Kim YT, Choi EK, Cho NH, Ko JH, Yang WI, Kim JW, et al. Expression of cyclin E and p27 in cervical carcinoma. Cancer Lett. 2000;153:41–50.
29. Coqueret O. New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment. Trends Cell Biol 2000;13:65–70.
30. Fillies T, Woltering M, Brandt B, Van Diest JP, Werkmeister R, Joos U, Buerger H. Cell cycle regulating proteins p21 and p27 in prognosis of oral squamous cell carcinomas. Oncol Rep. 2007 Feb;17(2):355-9.
31. Tamura N, Dong Y, Sui L, et al. Cyclin-dependent kinase inhibitor p27 is related to cell proliferation and prognosis in laryngeal squamous cell carcinomas. J Laryngol Otol. 2001;115:400–406.
32. Venkatesan TK, Kuropkat C, Caldarelli DD, et al. Prognostic significance of p27 expression in carcinoma of the oral cavity and oropharynx. Laryngoscope. 1999;109:1329–1333.
33. Perisanidis C et al. Evaluation of immunohistochemical expression of p53, p21, p27, cyclin D1, and Ki67 in oral and oropharyngeal squamous cell carcinoma. J Oral Pathol Med. 2012;41(1):40–46.
34. Kudo Y, Kitajjma S, Sato S, Miyauchi M, Ogawa I, Takata T. Establishment of an oral squamous cell carcinoma cell line with high invasive and p27 degradation activities from a lymph node metastasis. Oral Oncol. 2003 Jul;39(5):515-20.
35. Vallonthaiel AG, Singh MK, Dinda AK, Kakkar A, Thakar A, Das SN. Prognostic significance of cytoplasmic p27 in oral squamous cell carcinoma. J Oral Pathol Med. 2016 Jan.

36. Zhang M, Li J, Wang L, Tian Z, Zhang P, Xu Q, Zhang C, Wei F, Chen W. Prognostic significance of p21, p27 and survivin protein expression in patients with oral squamous cell carcinoma. Oncol Lett. 2013 Aug;6(2):381-386.
37. Pereira CS, Oliveira MV, Fraga CA, Barros LO, Domingos PL, Roy A, De-Paula AM, Guimarães AL. Impact of the epithelial dysplasia grading and Ki67 proliferation index in the adjacent non-malignant mucosa onrecurrence and survival in head and neck squamous cell carcinoma. Pathol Res Pract. 2012 Nov 15;208(11):651-6.
38. Woolgar J.A., Triantafyllou A. A histopathological appraisal of surgical margins in oral and oropharyngeal cancer resection specimens. Oral Oncology. 2005,41 (10):1034-43.
39. Kannan S, Chandran GJ, Pillai KR, Mathew B, Sujathan K, Nalinakumary KR, Nair MK. Expression of p53 in leukoplakia and squamous cell carcinoma of the oral mucosa: correlation with expression of Ki67. Clin Mol Pathol. 1996 Jun;49(3):M170-5.
40. Li Y, Li B, Xu B, Han B, Xia H, Chen QM, Li LJ. Expression of p53, p21(CIP1/WAF1) and eIF4E in the adjacent tissues of oral squamous cell carcinoma: establishing the molecular boundary and a cancer progression model. Int J Ora Sci. 2015 Sep 14;7(3):161-8. DOI: 10.1038/ijos.2015.5.
41. Nodin B, Fridberg M, Jonsson L, Bergman J, Uhlén M, Jirström K. High MCM3 expression is an independent biomarker of poor prognosis and correlates with reduced RBM3 expression in a prospective cohort of malignant melanoma. Diagn Pathol. 2012 Jul 17;7:82. DOI: 10.1186/1746-1596-7-82.
42. Carreón-Burciaga RG, González-González R, Molina-Frechero N, Bologna-Molina R. Immunoexpression of Ki-67, MCM2, and MCM3 in Ameloblastoma and Ameloblastic Carcinoma and Their Correlations with Clinical and Histopathological Patterns. Disease Markers. 2015. DOI 683087/2015.
43. Sobin LH, Wittekind CH. TNM classification of malignant tumours. New York. 7 ed., 2009.
44. Barnes L; Eveson JW, Reichart P, Sidransky D. Oral cavity and oropharynx. In: World Health Organization Classification of Tumours. Pathology and Genetics of Head and Neck Tumours. IARC Press. Lyon, 163-208, 2005.

Tables and figures

Table 1 Association between Ki67 immunostaining and clinicopathological variables

Clinicopathological characteristic	Ki67				*p-value*[a]
	Negative	(%)	Positive	(%)	
N =51	11	21,57	40	78,43	
Age					
<= 40 years	1	1,96	2	3,92	0,610
> 40 years	10	19,61	38	74,51	
Gender					
Female	3	5,88	15	29,41	0,530
Male	8	15,69	25	49,02	
Smoker					
No	4	7,84	15	29,41	0,945
Yes	7	13,73	25	49,02	
Alcohol intake					
No	6	11,76	19	37,25	0,679
Yes	5	9,80	21	41,19	
Primary site					
Tongue/Floor of the mouth	7	13,73	16	31,37	0,163
Others	4	7,84	24	47,06	
Size tumor (T)					
1 or 2	7	13,73	19	37,25	0,343
3 or 4	4	7,84	21	41,18	
Lymph node metastasis (N)					
0 or 1	11	21,57	32	62,75	0,106
2 or 3	0	0,00	8	15,68	
Stage					
I or II	7	13,73	16	31,37	0,163
III or IV	4	7,84	24	47,06	
Histological Grading[b]					
Well differentiated	2	15,38	11	16,67	
Moderately differentiated	6	46,16	29	43,94	0,987
Poorly differentiated	5	38,46	26	39,39	

[a] Chi-square test; [b] $n = 79$

Table 2 Association between MCM3 immunostaining and clinicopathological variables

Clinicopathological characteristic	MCM3				p-value[a]
	Negative	(%)	Positive	(%)	
N =51	7	13,73	44	86,27	
Age					
<= 40 years	0	0,00	3	5,88	0,476
> 40 years	7	13,73	41	80,39	
Gender					
Female	3	5,88	21	41,18	0,811
Male	4	7,84	23	45,10	
Smoker					
No	1	1,96	16	31,37	0,250
Yes	6	11,76	28	54,91	
Alcohol intake					
No	2	3,92	26	50,99	0,132
Yes	5	9,80	18	35,29	
Primary site					
Tongue/Floor of the mouth	4	7,84	20	39,22	0,565
Others	3	5,88	24	47,06	
Size tumor (T)					
1 or 2	3	5,88	23	45,10	0,643
3 or 4	4	7,84	21	41,18	
Lymph node metastasis (N)					
0 or 1	5	9,80	36	70,59	0,520
2 or 3	2	3,92	8	15,69	
Stage					
I or II	3	5,88	21	41,18	0,811
III or IV	4	7,84	23	45,10	
Histological Grading[b]					
Well differentiated	1	12,50	14	19,72	
Moderately differentiated	4	50,00	25	35,21	0,698
Poorly differentiated	3	37,50	32	45,07	

[a] Chi-square test; [b] $n = 79$

Table 3 Association betweenP27 immunostaining and clinicopathological variables

Clinicopathological characteristic	P27				p-value[a]
	Negative	(%)	Positive	(%)	
N =51	12	23,53	39	76,47	
Age					
<= 40 years	0	0,00	2	3,92	0,424
> 40 years	12	23,53	37	72,55	
Gender					
Female	3	5,88	14	27,45	0,484
Male	9	17,65	25	49,02	
Smoker					
No	4	7,84	10	19,61	0,602
Yes	8	15,69	29	56,86	
Alcohol intake					
No	6	11,76	14	27,45	0,382
Yes	6	11,76	25	49,03	
Primary site					
Tongue/Floor of the mouth	5	9,80	17	33,33	0,906
Others	7	13,73	22	43,14	
Size tumor (T)					
1 or 2	7	13,73	16	31,37	0,292
3 or 4	5	9,80	23	45,10	
Lymph node metastasis (N)					
0 or 1	9	17,65	34	66,67	0,310
2 or 3	3	5,88	5	9,80	
Stage					
I or II	5	9,80	15	29,41	0,842
III or IV	7	13,73	24	47,06	
Histological Grading[b]					
Well differentiated	3	14,29	12	20,69	
Moderately differentiated	12	57,14	25	43,10	0,537
Poorly differentiated	6	28,57	21	36,21	

[a] Chi-square test; [b] $n = 79$

Fig.1

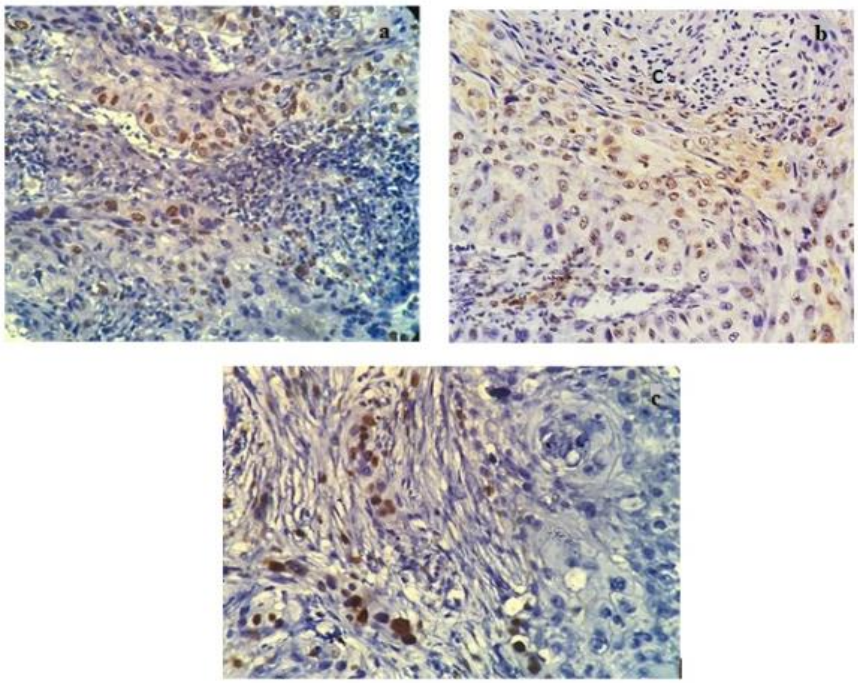

a
b
c
c

Fig. 2

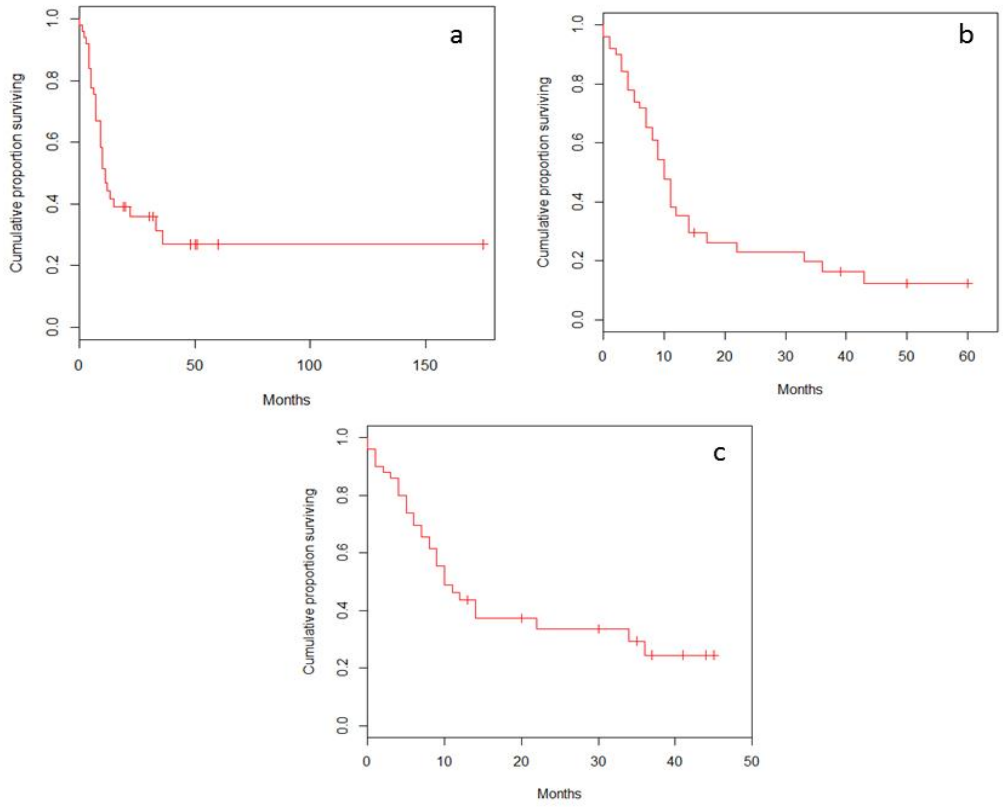

a
Cumulative proportion surviving
0.0
0.2
0.4
0.6
0.8
1.0
0
50
100
150
Months
b
Cumulative proportion surviving
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
60
Months
c
Cumulative proportion surviving
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
Months

Fig. 3

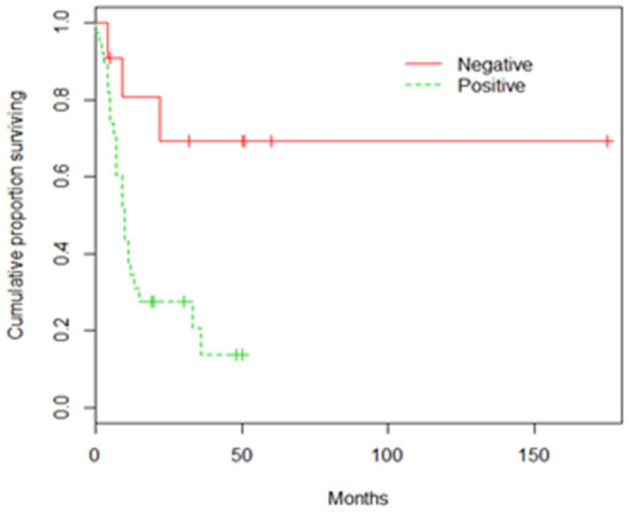

Negative
Positive
Cumulative proportion surviving
0.0
0.2
0.4
0.6
0.8
1.0
0
50
100
150
Months

Fig. 4

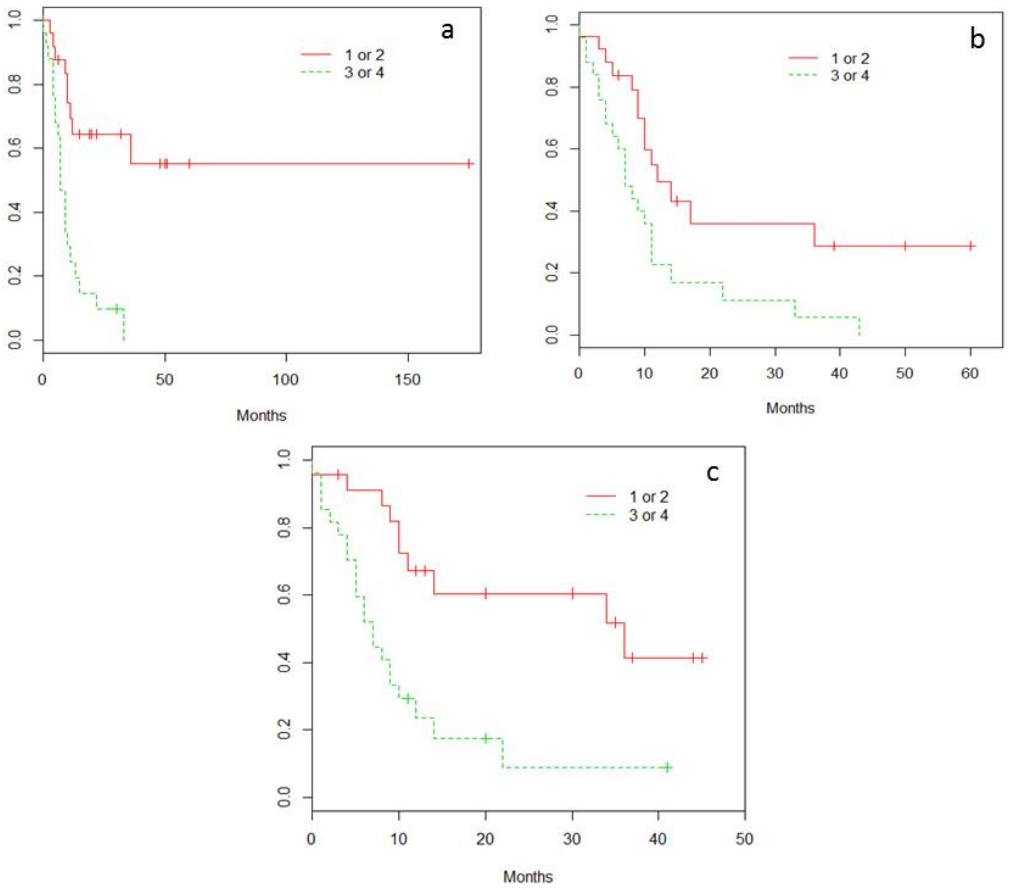

a
1 or 2
3 or 4
0.0
0.2
0.4
0.6
0.8
1.0
0
50
100
150
Months
b
1 or 2
3 or 4
0
10
20
30
40
50
60
Months
c
1 or 2
3 or 4
0
10
20
30
40
50
Months

Fig. 5

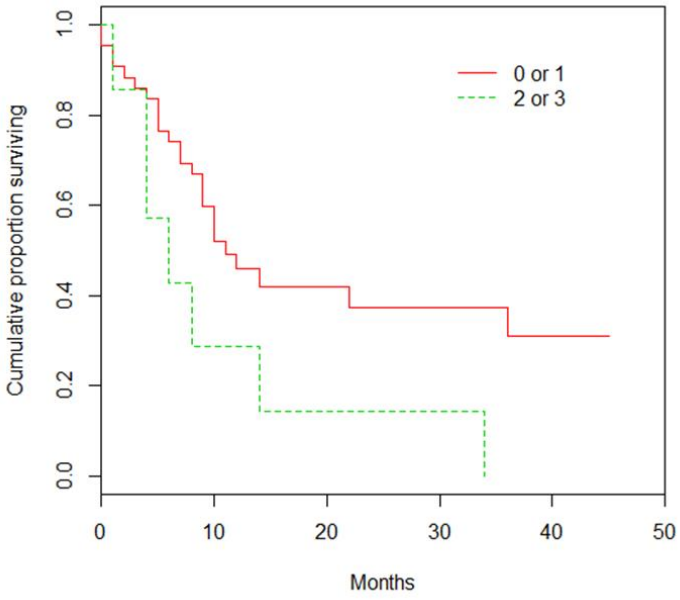
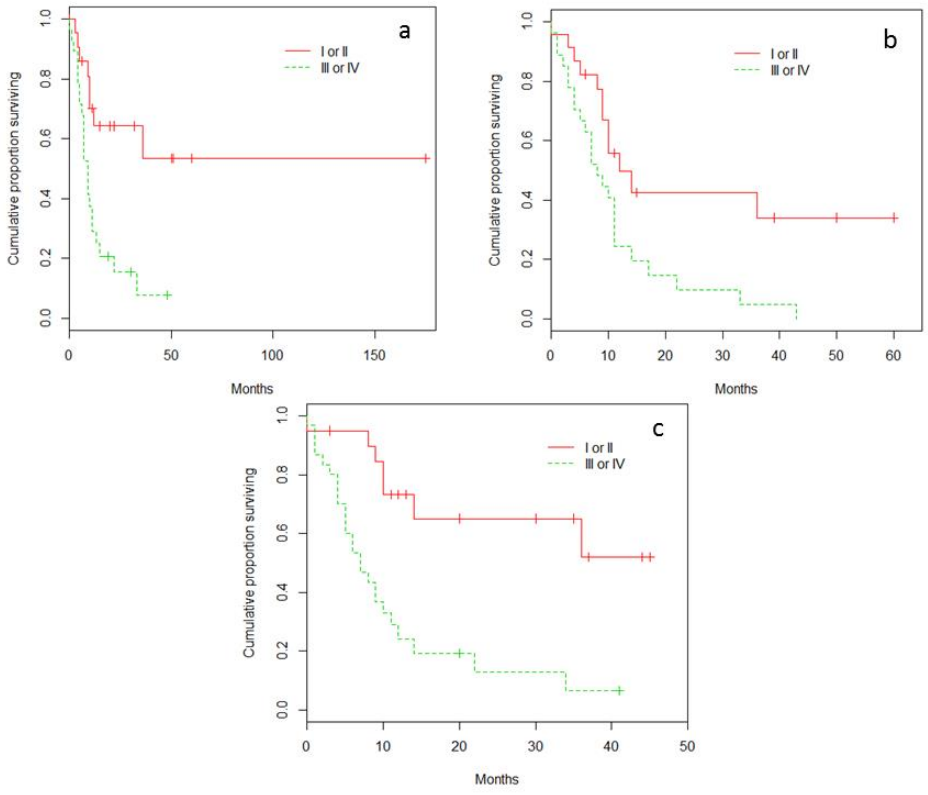

Cumulative proportion surviving
0.0
0.2
0.4
0.6
0.8
1.0
0 or 1
2 or 3
0
10
20
30
40
50
Months

Fig. 6


a
Cumulative proportion surviving
I or II
III or IV
0
50
100
150
Months
b
Cumulative proportion surviving
I or II
III or IV
0
10
20
30
40
50
60
Months
c
Cumulative proportion surviving
I or II
III or IV
0
10
20
30
40
50
Months