JUSCELINO DE FREITAS JARDIM

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“ANALÍSE CLINICOPATOLÓGICA E EXPRESSÃO DE FATORES DE PROLIFERAÇÃO CELULAR EM CARCINOMAS DE CÉLULAS ESCAMOSAS DE BOCA EM ESTÁDIO CLINICO AVANÇADO”

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Universidade Estadual De Campinas

Faculdade de Odontologia De Piracicaba

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Mestre em Estomatopatologia, área de Estomatologia

Dissertation presents to the Piracicaba Dentistry School of the University of Campinas in partial fulfillment of the requirements to obtained the degree of Master in Estomatopatologia, area of Stomatology.

Orientador: Prof. Dr. Luiz Paulo Kowalski

Este exemplar corresponde à versão final da tese defendida pelo aluno Juscelino de Freitas Jardim, orientada pelo Prof. Dr. Luiz Paulo Kowalski.

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Prof. Dr. LUIZ PAULO KOWALSKI

Prof. Dr. JOSÉ MAGRIM

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common malignancy in the oral cavity, accounting for almost 95% of these injuries, and about 38% of malignant tumors of the head and neck. More than 50% of all patients have advanced disease at the time of diagnosis, factor that reflects in low survival rates at 5 years. Histopathological factors such as vascular and perineural invasion have been associated with low rates of recurrence and survival. Moreover, patients with the same site and similar histology may have different biological behaviors, due to their differing biological characteristics and therefore exist an interest in the identification of biomarkers that could be used in the clinical practice in order to better prognosticate and risk-stratify patients. The aim of this study was to evaluate the prognostic significance of the perineural invasion (PNI) and lymphovascular invasion (LVI) as well as the expression of Mcm-2, Cyclin D1, Ki-67 and p53 in advanced stage OSCC. A retrospective review of patients with OSCC of the tongue and floor of mouth in advanced clinical stage, treated by surgery in the Department of head and neck of the AC Camargo Cancer Center between the years 1998 and 2009 was conducted. Of 142 eligible cases, 88 paraffin-embedded tissue were rescued from the Department of Pathology of the same institution and immunohistochemistry reactions were performed for markers previously described. All slides have gone digitalized through the equipment Aperio System (Vista, CA, USA). Quantifications were performed from Imagescope software (Aperio System, USA) and statistical tests with significance level of 5% were taken to access correlations with prognosis. Our results showed that both PNI (p<0.001) as LVI (p=0.01) had negatively influence on overall survival of the patients. Other factors as T stage (p=0.003), positive lymph node (p=0.002) and extracapsular nodal spread (p<0.001) also had prognostic impact to predict poor survival. In relation to the proliferative markers, we found that high expression of Mcm-2 (p<0.001) and Cyclin D1 (p=0.005) had strong association with low rates of overall survival, whereas p53 and Ki-67 did not reach significance with this parameter. Mcm-2 was also correlated with recurrence (p=0.025). Cyclin D1 and p53

were significance with the N stage. In conclusion, the increasing expression of Mcm-2 and Cyclin D1 showed important correlation with prognosis and survival as well as histopathological features, such as PNI.

Keywords: squamous cell carcinoma, prognosis, biomarkers, cancer, oral cavity.

RESUMO

O carcinoma de células escamosas (CEC) é a neoplasia maligna mais frequente na cavidade bucal, correspondendo a quase 95% destas lesões, e cerca de 38% dos tumores malignos de cabeça e pescoço. Mais de 50% dos portadores deste tipo de tumor apresentam estágio avançado da doença no momento do diagnóstico, fator que reflete em baixas taxas de sobrevidas em 5 anos. Fatores histopatológicos como invasões perineural e vascular têm sido relacionadas com recorrência e baixas taxas de sobrevida. Ainda, pacientes com mesmo sítio de acometimento e histologia tumorais semelhantes podem ter comportamentos biológicos distintos, frente a isso, a importância da busca por biomarcadores para predizer prognóstico e risco estratificado. O propósito deste estudo consistiu em avaliar o significado clínico e prognóstico dos fenômenos de invasão perineural (IP) e invasão vascular (IV) bem como da imunoexpressão de Mcm-2, Ciclina D1, Ki-67 e p53 em CECs de língua e assoalho em estádio clínico avançado. Foi realizado um levantamento retrospectivo de pacientes com CEC de língua e assoalho de boca em estádio clínico avançado, tratados previamente por cirurgia no departamento de cirurgia de cabeça e pescoço do AC Camargo Cancer Center entre os anos de 1998 e 2009. De 142 casos elegíveis para o estudo, 88 blocos de parafina foram resgatados do departamento de Patologia da mesma instituição e reações de imunoístoquímica foram realizadas para os marcadores já descritos anteriormente. Todas as lâminas passaram por um processo de digitalização através do equipamento Aperio System (Vista, CA, USA). Quantificações das marcações foram obtidas através do software Imagescope (Aperio System, USA) e testes estatísticos com nível de significância de 5% foram tomados para acessar correlações com prognóstico. Os resultados mostraram que tanto IP ($p< 0,001$) como IV ($p= 0,01$) influenciaram negativamente a sobrevida em 5 anos dos pacientes. Outros fatores como tamanho tumoral (estádio T) ($p=0,003$), estádio N+ ($p= 0,002$) e ruptura de cápsula linfonodal ($p< 0,001$) também obtiveram impacto em predizer sobrevida. Com relação aos marcadores de proliferação celular, altas taxas de Mcm-2 ($p<0,001$) e Ciclina D1 ($p = 0,005$) tiveram relação direta com

taxas de sobrevida global menores que 5 anos, enquanto p53 e Ki-67 não alcançaram significância. Mcm-2 também foi altamente relacionado com recorrência (*p*=0,025), enquanto Ciclina D1 e p53 foram correlacionados com estádio N. Em conclusão, o aumento na expressão de Mcm-2 e Ciclina D1 exibem importante correlação com prognóstico e sobrevida dos pacientes, assim como fatores histopatológicos como invasões perineural.

Palavras-chave: carcinoma de células escamosas, biomarcadores, fatores prognósticos, neoplasia, boca.

SUMÁRIO

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"De tudo, ficam três coisas: A certeza de que estamos sempre começando, a certeza de que precisamos continuar, e a certeza de que seremos interrompidos antes de terminar. Portanto, devemos fazer da interrupção um caminho novo; da queda, um passo de dança; do medo, uma escada; do sonho, uma ponte; da procura, um encontro."

(Fernando Pessoa)

INTRODUÇÃO

A Organização Mundial de Saúde (2005) define câncer como um grupo de doenças que podem afetar qualquer parte do corpo, sendo caracterizado pela rápida progressão de células anormais que crescem além de seus limites habituais, e que podem então invadir estruturas adjacentes e metastatizar para outros órgãos.

O Instituto Nacional do Câncer (INCA) estimou, para o ano de 2014, aproximadamente 576.000 casos novos de câncer no Brasil. Desse total, 15.290 (2,65%) acometeriam a cavidade oral (boca e orofaringe), sendo o quinto tipo mais frequente no gênero masculino e o décimo segundo no feminino. Esses valores correspondem a um risco estimado de 11,54 casos novos a cada 100 mil homens e 3,92 a cada 100 mil mulheres (Ministério da Saúde 2014).

A carcinogênese oral é um processo multifásico que requer a desestabilização de vários sistemas que controlam e coordenam o comportamento e homeostase celular (Todd *et al.,* 2002), havendo ruptura da sinalização celular, reparo do DNA e alteração do ciclo celular (Bettendorf *et al*., 2004). Segundo Boshoff & Weiss (2001), ocorre na maioria dos casos, um acúmulo sequencial de mutações somáticas por muitos anos, antes da expansão clonal de células malignas.

O desenvolvimento do CEC relaciona-se diretamente a alterações na estrutura e regulação genética, principalmente naqueles genes reguladores do processo de divisão celular (Peltonen *et al*., 2010). Pich *et al*. (2004) citaram que dentre as alterações, as mutações do gene P53 e Ki67 são as mais comuns, podendo ser utilizadas como indicadores de prognóstico nos pacientes com carcinomas de boca. Alterações como perda de heterozigose, instabilidade de microsatélites e repetições de sequências também contribuem para a ocorrência de tais alterações genéticas (Kodani *et al.,* 2001).

O tipo histológico mais comum de câncer bucal é o carcinoma CEC, também chamado carcinoma epidermóide ou carcinoma espinocelular pelo fato da proliferação celular originar-se da camada espinhosa do epitélio.

Corresponde a 95% de todos os tumores malignos da boca, cerca de 38% dos tumores malignos situados na região de cabeça e pescoço (Scully, 2009).

Já está bem estabelecido que o câncer de boca possui etiologia multifatorial, fazendo parte tanto fatores intrínsecos quanto extrínsecos, e sendo necessário a ação de mais de um fator desencadeante para a produção de malignidade (Scully, 2009; Nagpal *et al.*, 2003). Tabagismo e o etilismo crônico são os principais fatores de risco, uma vez que apenas 15 a 20% dos portadores de lesões malignas bucais não tem história pregressa destes hábitos (Warnakulasuriya, 2009; Bettendorf *et al.*, 2004). Ainda, o risco de desenvolvimento desta neoplasia na população geral é aumentada em cerca de sete vezes com o tabagismo, e em até 15 vezes quando este hábito está associado ao consumo crônico de álcool (Kruse *et al.*, 2010). Fatores intrínsecos tais como alterações genéticas, deficiências nutricionais e imunossupressão; e fatores extrínsecos como radiação solar e alguns vírus também são considerados agentes etiológicos da doença (Nagpal *et al.,* 2003).

A família do Papiloma Vírus Humano (HPV) conta com mais de 100 subtipos virais catalogados (van Monsjou et al., 2013), e sua infecção está presente em aproximadamente 5,2% de pacientes com câncer no mundo inteiro, incluindo tumores de ânus, trato genital e nos últimos anos tem ganhado destaque pela relação de casos localizados na orofaringe (Chung et al., 2013) sendo a exposição, particularmente, aos subtipos 16 e 18 bem reconhecidos nestes casos.

Os portadores de CEC oral são especialmente pacientes do gênero masculino entre a 5º e a 8º décadas de vida. No entanto, observa-se atualmente um aumento na incidência desta neoplasia em indivíduos jovens, com menos de 45 anos de idade e que muitas vezes não foram expostos aos fatores de risco ambientais mais significativos (tabaco e álcool) (Warnakulasuriya, 2009).

O tumor tem apresentação clínica variada, apresentando-se clinicamente de acordo com o tempo de evolução da doença. Lesões iniciais podem se apresentar como leucoplasias, eritroleucoplasias ou úlceras. A neoplasia pode se apresentar com aspecto exofítico ou endofítico, mostrando às vezes

aparência nodular. Língua (borda lateral) e assoalho bucal são as localizações bucais mais comuns de acometimento (Scully, 2009).

As neoplasias malignas assim com o CEC, exibem caracteristicamente um crescimento infiltrativo, sendo capazes de invadir tecidos vizinhos, ganhar uma via de disseminação, chegar a sítios distantes (metástases) (Takes *et al*., 2012). Tal fenômeno é um processo complexo e é considerado um evento tardio na carcinogênese que se inicia com a proliferação celular, havendo posteriormente perda do contato com células vizinhas, migração através da matriz intersticial, invasão de vasos linfáticos e sanguíneos e crescimento em linfonodos e órgãos distantes. Dentre as localizações anatômicas o câncer de língua apresenta um alto potencial de invasão, e grande probabilidade de desenvolver metástase para linfonodos regionais (Kowalski *et al*., 1993).

A agressividade destes tumores está relacionada a diversos fatores, dentre os quais cita-se o grau histológico de malignidade, tamanho da lesão, grau de comprometimento dos tecidos vizinhos, presença de metástase no momento do diagnóstico e localização anatômica do tumor (Woolgar, 2007; Massano *et al.,* 2006). Parâmetros clínicos e imaginológicos como tamanho tumoral e disseminação metastática, consistem em excelentes indicadores prognósticos do paciente. Estes parâmetros permitem o estadiamento das neoplasias malignas através do sistema TMN, onde T se refere ao tamanho do tumor (variando de T1 a T4), N à propagação aos linfonodos regionais (N0 a N3) e M à metástase à distância (M0 e M1). De acordo com os parâmetros estabelecidos pode-se classificar as lesões em estádios clínicos de I a IV (UICC*,* 2009). O prognóstico do câncer oral se mostra variável, com taxas de sobrevida de 5 anos, oscilando entre 74%, para lesões iniciais, e 29%, para carcinomas diagnosticados em estádio IV (Carvalho et al., 2004).

Um dos maiores desafios na pesquisa em câncer é o desenvolvimento de terapia capaz de impedir a disseminação tumoral e metástase, pois estes eventos diminuem drasticamente as chances de cura e sobrevida dos pacientes. A maioria dos tumores sólidos apresenta metástase para os linfonodos regionais, preferencialmente via vasos linfáticos, que geralmente é o primeiro sinal de disseminação cancerígena (Takes *et al*., 2012). No entanto, tumores malignos também têm a habilidade de induzir o crescimento de novos

vasos sanguíneos a partir de vasos periféricos (angiogênese), que são importantes para a progressão tumoral, crescimento, agressividade e habilidade para produzir metástases (Folkman, 1995; Massano *et al.,* 2006). A estrutura desorganizada e tortuosa dos neovasos permite a invasão por células malignas. Ramificações dos vasos pré-existentes comunicam com a rede vascular imatura possibilitando a migração destas células. Estas seguem, assim na circulação sanguínea, podendo originar metástases à distância.

Segundo Woolgar (2006) a avaliação do espécime cirúrgico fornece importantes informações a respeito da agressividade do tumor, e que implicam diretamente com o prognóstico dos pacientes. Os principais fatores prognósticos reconhecidos são: dediferenciação, invasão de estruturas, ruptura linfonodal, invasão linfática e vascular e invasão perineural.

A invasão linfovascular é definida por Sutton et al. (2003) como a presença de agregados de células tumorais no interior de vasos linfáticos ou infiltrando seu endotélio. A disseminação de células tumorais via vasos linfáticos e suas implicações no tratamento e prognóstico dos pacientes tem sido estudadas há anos (Jones *et al.,* 2009). As características próprias dos capilares linfáticos, como a descontinuidade da membrana basal, facilitam a permeação de células malignas. Outra característica dos vasos linfáticos como a baixa velocidade do fluxo linfático e a composição da linfa (semelhante à do fluido intersticial) favorecem a viabilidade celular. Como tal, a corrente linfática é preferencial para a disseminação de muitos tumores malignos, principalmente os que apresentam histogênese epitelial.

Por sua vez, a invasão perineural é definida como um tropismo das células tumorais para feixes de nervos nos tecidos circundantes. Muitos trabalhos tem reportado a associação entre este parâmetro com recorrência, metástase linfonodal e sobrevida (Fagan et al., 1998, Binmadi & Basile, 2011), porém seu real valor prognóstico permanece em discussão.

Para avaliar o grau de proliferação celular, alguns biomarcadores relacionados têm sido amplamente empregados com a finalidade de obter uma correlação entre este fenômeno e seu impacto na sobrevida dos pacientes. O Ki-67 é um marcador de proliferação celular, altamente expresso na maioria dos tumores, que identifica um antígeno expresso nas fases G1, S e G2 do

ciclo celular; o aumento de sua expressão foi associado à presença de metástase linfonodal, (Coutinho-Camillo et al. 2010) e seu índice tem sido correlacionado com alto risco de recorrência, bem como da agressividade do tumor (Silva et al. 2008) e pior prognóstico em pacientes com CEC de língua (Xie et al. 1999; Silva et al. 2008) e boca (Myoung et al. 2006; Coutinho-Camillo et al. 2010).

Outro marcador relacionado a proliferação celular, p53, é uma proteína, expressa pelo gene TP53 que se encontra no cromossomo 17, e funciona como inibidor da divisão celular na fase G1 do ciclo celular, podendo induzir apoptose, sendo responsável por manter a integridade do genoma. A mutação do gene TP53 é uma das mais conhecidas e mais freqüentes alterações genéticas identificadas em tumores malignos (Peltonen et al., 2010; Massano et al. 2006; Sarkis et al. 2010). O aumento da expressão de p53 está associado a um maior número de metástases e está correlacionada com um prognóstico ruim (Oliveira et al. 2007).

Ainda, a proteína mantenedora do minicromossomo 2 (Mcm-2) é uma das seis proteínas (Mcm-2-7) que se unem no complexo pré-replicativo e são essenciais para a replicação do DNA em células eucarióticas (Kodani *et al*., 2003). Alterações nos níveis de uma única proteína do complexo Mcm levam à ruptura da estabilidade genômica e, algumas proteínas implicadas diretamente na patogênese de alguns tipos de carcinomas modulam a atividade deste complexo de proteínas (Kato *et al*., 2003). Todas as seis proteínas deste complexo são abundantes através do ciclo celular e são quebradas mais rapidamente na diferenciação e mais lentamente na quiescência (Wojnar et al., 2010). Por esta razão, os anticorpos contra estas proteínas têm a capacidade de detectar mais células no ciclo celular do que outros marcadores de proliferação como o antígeno Ki-67 (Szelachowska *et al*., 2006). Por apresentarem estas características, a avaliação da expressão das proteínas Mcm, através da imunoístoquimica, vem se tornando marcador promissor na avaliação da replicação celular em diferentes tumores.

Gakiopoulou *et al*. (2007), utilizando anticorpos monoclonais para as proteínas Mcm-2 e Mcm-5 em um grupo de pacientes portadoras de adenocarcinoma de ovário, observaram que a expressão destas proteínas

apresentava associação com o grau tumoral, presença de doença residual e boa correlação com o índice Ki-67. A utilização da expressão da proteína Mcm-2 em carcinoma de mama também evidenciou uma associação positiva com o grau histológico do tumor (Wojnar et al., 2010). Gueiros et al. (2011) reportaram que altas taxas de Mcm-2 em carcinomas de língua tinha íntima relação com metástase linfonodal, e afeta negativamente a sobrevida global destes pacientes.

Outro marcador relacionado que tem sido investigado nos últimos anos é a Ciclina D1 que é uma molécula reguladora do ciclo celular no controle da fase G1 para fase S e tem sido associada ao aumento do risco de metástases linfonodais em câncer de língua (Bova *et al.,* 1999) e boca, além de ser um fator prognóstico (Massano *et al.,* 2006).

Carlos de Vicente *et al.* (2001), evidenciaram uma forte correlação entre superexpressão de Ciclina D1 com metástases linfonodais e estádios avançados da doença ($p < 0.001$) em CECs orais. Mineta *et al*., (2000) relataram que a alta expressão de Ciclina D1 em CECs de boca é associado com pobre sobrevida e progressão para ruptura de cápsula dos linfonodos metastáticos.

Embora bastante estudado, o CEC oral ainda permanece com muitos aspectos não compreendidos, principalmente no que concerne ao prognóstico. Em virtude disto, nosso trabalho objetivou avaliar a influência prognóstica de fatores histopatológicos encontrados nos espécimes cirúrgicos de pacientes submetidos à cirurgia radical como tratamento inicial para CECs de boca em estádio clínico avançado, bem como a associação entre a expressão de proteínas do ciclo celular e seu impacto na sobrevida dos mesmos.

CAPÍTULO 1

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PROGNOSTIC IMPACT OF PERINEURAL AND LYMPHOVASCULAR INVASIONS IN ADVANCED STAGE ORAL SQUAMOUS CELL CARCINOMA

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Abstract

Perineural and lymphovascular invasions (PNI and LVI) have been associated with the risk of local recurrences and lymph node metastasis. This study aimed to evaluate the prognostic impact of PNI and LVI in patients with advanced

stage squamous cell carcinoma of the tongue and floor of the mouth. One hundred forty-two patients without previous treatment were selected. The included patients underwent radical surgery with neck dissection and adjuvant treatment. Clinicopathological informations were retrieved from medical charts including the histopathological and surgical reports. Univariate analysis was performed for assessment to impact of studied variables on survival. Overall survival was negatively influenced by six tumour-related factors: increasing T stage (p=0.0032), more than two clinically positive nodes (p=0.002), extracapsular spread of lymph node metastasis (p<0.001), tumour thickness (p=0.04) perineural invasion (p<0.001) and lymphovascular invasion (p=0.012). Disease-free survival were influenced by perineural invasion (p=0.04), extracapsular spread of lymph node metastasis (p=0.008) and N stage (p=0.006). The multivariate analysis showed that PNI was independent predictive factor for overall survival (p=0.03) and disease-free survival (p=0.01). Thus the presence of PNI in oral carcinoma surgical specimens has a significant impact on survival outcomes in patients with advanced stage oral squamous cell carcinoma submitted to radical surgery and adjuvant radio/radiochemotherapy.

Keywords: Oral Squamous Cell Carcinoma, Oral Cancer, Prognosis, Survival, Lymphovascular Invasion; Perineural Invasion

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common human cancer accounting for over 500,000 new cases annually worldwide, and more than 50% of all patients have advanced disease at the time of diagnosis[1]. With the advent of multimodality treatment programs that include surgery, radiotherapy and chemotherapy, several reports show that patients with stage III and stage IV (OSCC) are surviving for a longer period. However, mortality rates in population-based registries reported by population-based registries remain nearly unchanged, motivating the search for prognostic factors aiming to tailor the individual management of patients[2-4].

In the search for prognostic factors in OSCC many variables have been identified and can broadly be placed in the categories of tumour related, patient related and treatment related factors[5]. Of the known prognostic factors, TNM stage, histologic grade, and tumour thickness are almost universally recognized; however, the prognostic value of other clinicopathological factors is often uncertain and controversial[4-6]. Among the various parameters used to predict the outcome of malignant disease in OSCC, the lymphovascular invasion (LVI) and perineural invasion (PNI) are both in wide use as indicator of aggressive behavior[1-2, 7].

PNI is a tropism of tumour cells for nerve bundles in the surrounding tissues. PNI is well known as an independent predictor of poor outcome in colorectal carcinoma and salivary gland malignancies. However in OSCC there is no consensus among the authors about the real prognostic impact[7-9]. On the other hand LVI was classified according to the presence or absence of neoplastic cells, located in the wall or lumen of blood or lymphatic vessels. The LVI has been demonstrate as a good tool, and was correlated with low rates of survival and high risk of recurrences[10]. However, due difficult to define and recognize with certainty this parameter, some grading systems have been omitting this in their systems[2].

The aim of this study was to evaluate the prognostic impact of histopathological features with emphasis in perineural and lymphovascular invasions on survival of patients with advanced stage OSCC.

PATIENTS AND METHODS:

A retrospective review of medical records was performed for 142 patients with advanced stage (CS III-IV) SCC of the oral tongue and floor of the mouth treated primarily with surgery at the A C Camargo Cancer Center, São Paulo, Brazil. The eligibility criteria included previously untreated patients, submitted to treatment in the institution from 1998 to 2009. This study obtained approval by the Institutional Ethics Committee on Research (Protocol # 1684/12).

All patients had received primary surgical tumour resection with simultaneous neck dissection and adjuvant radio/radiochemotherapy. Patients with distant metastases (M1) at the beginning of the treatment were not

included in this study as well as unresectable tumours and patients that had any prior treatment. TNM restaging was reviewed according to the International Union Against Cancer Criteria (UICC, 2002)[14].

For all cases the medical and pathological charts were examined for demographic data (age, gender, and race), clinicopathological information (clinical stage, invasion of adjacent structures, lymphovascular invasion, perineural invasion, histological grade, pattern of invasion, tumour size, nodal status, and margins).

Twenty-three variables were analysed by univariate analysis to assess their influence on survival. Survival calculations were determined by the Kaplan-Meier method and compared using the log-rank test. Overall survival was defined as the time from the beginning of primary therapy to death from any cause. For multivariate analysis, Cox proportional hazards model was used to estimate the independent prognostic impact of patient and tumour related factors on survival. All statistical analyses were performed using R, version 3.0.1 (R Development Core Team (2010), Viena, Austria, www.R-project.org). P values less than 0.05 were considered statistically significant.

RESULTS

Among 142 eligible patients, 109 (76.8%) were male, and 33 (23.2%) were female. Most of the patients reported smoking (113 patients, 79.6%) and drinking alcohol (89 patients, 62.7%). The patients' mean age was 57 years. In 68.3% (97 cases) of cases the tongue was the primary site, while 31.7% (45 cases) tumour arised in the floor of the mouth. Ninety-two (64.8%) patients had their histological grade defined as well differentiated, whereas 50 (35.2%) were classified as moderately/poor differentiated. The clinicopathological data are summarized in *table 1*.

Of the 142 patients 38 (26.8%) were node negative, 53 (37.2) had N1 disease, 22 (15.5) were classified as N2a, 15 (10.5%) as N2b, 10 (7%) as N2c, and 4 (2.8%) as N3. A total of 68 (47.9%) patients were classified in Stage III, whereas 74 (52.1%) in stage IV. LVI was present in the primary tumour in 58 (40.8%) of documented cases, while PNI was present in 71(50%) cases of our sample. Of the 93 patients with positive node positive nodes at pathological

examination (pN+) there were 47 that had LVI and 50 that exhibited PNI. Therefore, the percentages of LVI and PNI with positive nodal disease were 50.5% and 53.8%, respectively. There were 104 (73.4%) recurrences, and of then 48 (46.2%) had LVI. Only 10 (9.6%) patients with LVI had not showed recurrence. Concerning to PNI, 59 patients (56.7%) with invasion had recurrences, while 10 (9.6%) remained with no evidence of disease.

The median follow up was of 31.2 months (range 2 to 176 months). The disease free survival was estimated in 25% for 5 years. Overall survival at 5 years was of 42.2% and in 10 years it was of 28.8%.

The overall survival rates were negatively influenced by six tumour-related factors by univariate analysis: Increasing T stage (p=0.0032), more than two clinically positive nodes (p=0.002), extracapsular spread of lymph node metastasis (p<0.001), tumour thickness (p=0.04) PNI (p<0.001) and LVI (p=0.012). Disease-free survival rates were positively influenced by perineural invasion (p=0.04), extracapsular spread of lymph node metastasis (p=0.008) and N stage (p=0.006).

In addition, LVI had correlated with histological grade (p=0.03) and lymph node capsular rupture (p=0.034), while PNI had relation with the pathological lymph node metastases (p=0.019). The association between LVI/PNI and clinicopathological parameters is shown in *table 2*.

Results from the survival analysis using the Cox proportional hazard model showed that PNI was significant independent predictor for overall survival (p=0.017), and also for disease-free survival (p=0.032), whereas LVI did not reach significance in multivariate analysis on survival outcomes (*Tables 3 and 4*).

DISCUSSION

In agreement with the literature our findings showed that advanced stage OSCC cases are diagnosed in male older than 55 years[6, 11-12]. However, the incidence appears to be increasing in women especially those who smoke and heavily consume alcohol[13].

The TNM system is used to categorize the malignancy in any site of the body. These are T is the ability to grow and locally invade, N has relation with

the ability to metastasize to regional lymph nodes and M is the ability to metastasize to distant sites[14]. In oral cavity these three criteria are independent indicators of prognosis although they are inter-related. Increasing size by T stage leads to an increase in the rate of occult metastases[10,15-16] as well as increasing N stage is associated with the development of distant metastases, particularly with the multiple involvement or the presence of extracapsular spread[10]. A recent study showed that younger patients suffering from OSCC had a significantly more advanced N stage, more PNI, and higher rates of treatment failure and mortality when compared to an older patient population[12]. Still, younger patients (<40 years) can have a tendency towards more aggressive disease, and PNI can be a marker of aggressiveness[7-8], but our findings did not show this fact.

Our sample included only patients with resectable advanced stage OSCC affecting tongue and floor of the mouth as primary sites and of the 142 cases the 5-year overall survival was 42.3%. This data is in agreement with the 5-year survival rates compiled from the literature that is established as rate of 36% in advanced Head and neck cancer[4,16]. Locoregionally advanced squamous cell carcinoma of the oral cavity and oropharynx continue to be a major clinical problem despite advances in surgery, radiation, and chemotherapy[2, 16].

Among the histologic parameters in OSCC, perineural invasion has a controversial importance. PNI is defined as a tropism of tumour cells for nerve bundles in the surrounding tissues and is well known as an independent predictor of poor outcome in colorectal carcinoma and salivary gland malignancies[7, 18-19]. PNI is considered as a form of metastatic tumour spread similar to but distinct from vascular or lymphatic invasion that hinders the ability to establish local control of a malignancy because neoplastic cells can travel along nerve tracts far from the primary lesion and are often missed during surgery[2, 7, 20]

Some studies have shown that infiltration of the perineural space of nerves at the advancing front of the tumour is related to the tumour site and thickness, pattern of invasion at the advancing tumour front, presence of nodal metastasis and presence of capsular ruptute[2,21-22]. Our study demonstrated the

correlation between the presence of PNI and decreased overall survival (p<0.001). However PNI was not associated with the capsular rupture of the metastatic lymph nodes in contrast with other studies[9,21], but it was associated with the number of positive lymph nodes (p=0.019) and recurrence (p=0.009). Similar findings have been reported by Sutton *et al.*[22] and Binmadi e Basile[7] that classified the PNI as a significant prognostic indicator due to the ability of OSCC to spread to cervical lymph nodes and therefore should be considered for neck dissection and the use of adjunctive treatment. In last years the authors have discussed the role of PNI in OSCC, and most of published data have correlated this parameter with the development of metastases and survival[2,7-9]. However, there is no consensus of the real prognostic value of PNI in patients with OSCC[9].

LVI was defined as the presence of aggregates of tumour cells within endothelial lined channels or invasion of the media of a vessel with ulceration of the intima[2]. On oral carcinoma, LVI does not seem to have had as much attention[10] and many grading system omitted this characteristic since it is considered difficult to define and recognize with certainty[2]. LVI have a significant association with tumour site, diameter and thickness, perineural invasion, invasive front, multifactorial histological malignancy scores, pattern of invasion; nodal metastasis, status of resection margins, local recurrence, and survival[2,10, 21-22]. In common with these studies our findings on LVI reveled a significant relationship with metastatic lymph node capsular rupture and overall survival.

There is global consensus that nodal status is one of the most important prognostic factors and together with distant metastasis has a significant impact on survival rates[1-3, 7-9, 21]. In a study performed by Woolgar *et al.*[29] it was shown that patients with one positive cervical node without evidence of capsular rupture would have rates of survival in five years similar to those without evidence of nodal disease, whereas more than two nodes and extracapsular rupture are considered as poor prognostic predictors and have association with distant metastasis. For distant metastases, traditionally, cases initially staged as N2 or N3, and those with uncontrolled locoregional disease were thought to be most at risk[2]. This study showed that the outcome survivor of OSCC is affected

by increasing T stage (p=0.003), more than two clinically positive nodes (p=0.002), extracapsular spread of lymph node metastasis (p<0.001). These findings were also reported by other authorities[2-3,17].

The multivariate analysis showed that LVI was not an independent predictor of survival. Literature review revealed only one study that reported LVI with significance on multivariate analysis[10] and the authors discussed about the possibility of using this parameter as a criteria to define aggressiveness and to select patients for more specific and aggressive treatment in the future. The prognostic importance of LVI in other solid tumours is well established and has been correlated with development of distant metastases and survival[23,25]. However, patients with advanced tumours had a great tendency to develop distant metastases[20].

The Cox survival model showed that PNI was a significant independent prognostic factor for the disease-free survival and overall survival. According to Binmadi and Basile[7] OSCC is a neurotropic malignancy that traditionally has been difficult to treat. Evidence suggests that PNI is correlated with late stage disease[8]. There is a strong tendency toward neural invasion in late stage carcinoma but no association with early stage SCC of the tongue[15]. In concordance with these authors our high rates of PNI translated into a significant poor outcomes on survival and these findings corroborate with the literature that have been demonstrated of PNI in OSCC should impact adjuvant treatment decisions and surgical management of this disease[2, 7-8].

According to Woolgar[2] the assessment of the surgical resection specimen continues to provide information that is central to determining the post-operative treatment needs and prognosis for an individual patient with oral squamous cell carcinoma. Several authors have questioned the prognostic accuracy of the TNM system for oral cancer since neither patient's comorbidity, specific tumour related factors nor multimodal treatment regimens such as adjuvant radiochemotherapy (RCT) are incorporated. In the future several parameters as PNI and LVI must be included in grading systems, because their value has being shown in many studies in the literature, as an attempt to better classify the tumours according their biologic behavior and use this information for the indication of a specific modality of treatment.

Our results indicate that the presence perineural invasion in oral carcinoma surgical specimens has a significant impact on survival outcomes in patients with advanced stage tumours submitted to radical surgery and adjuvant radio/radiochemotherapy. Moreover, PNI was an independent factor to reduce survival outcomes.

Competing interests

The authors declare that they have no competing interests.

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Ethical Approval

This work was approved by the A.C. Camargo Cancer Center Ethics Committee (Protocol number 1684/12).

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Table 1. Clinicopathologial features of the sample.

Variables	Categories	Patients	%
Age	Range	22 – 82	
	Mean	57	
	Median	58	
Gender			
	Male	109	76.8
	Female	33	23.2
Tobacco Smoking			
	Yes	113	79.6
	No	12	8.5
	n/a	17	11.9
Alcohol consumption			
	Yes	89	62.7
	No	34	23.9
	n/a	19	13.4
Tumour Site			
	Tongue	97	68.3
	Floor of the mouth	45	31.7
Tumour Size			
	T1	6	4.2
	T2	37	26.3
	T3	54	38
	T4	45	31.5
Nodal Status			
	N0	38	26.8
	N1	53	37.2
	N2	47	33.2
	N3	4	2.8
Clinical Stage			
	Stage III	68	47.9
	Stage IV	74	52.1
Histological Grade			
	Well differentiated	92	64.8
	Moderately/poorly differentiated	50	35.2
Recurrence			
	Yes	104	73.2
	No	33	23.2
	n/a	5	3.6
Status			
	Alive	46	32.3
	Dead	93	65.4
	Lost to follow-up	3	2,3

*n/a information not available

Table 2. Correlation between LVI/PNI and clinicopathological parameters by univariate analysis

Clinicopathological parameters	Categories	LVI +	LVI -	*P* Value	PNI +	PNI -	*P* value
Gender							
	Male	42	67	NS	60	49	NS
	Female	16	17		11	22	
Age							
	<40	7	10	NS	8	9	NS
	>40	51	74		63	62	
Histological Grade							
	Well differentiated	35	57	**0.03**	47	45	NS
	Moderately/poorly differentiated	23	27		25	25	
Pathological lymph node metastasis	Yes	47	46	NS	50	43	**0.019**
	No	11	38		21	28	
Lymph node rupture							
	Yes	34	29	**0.034**	39	24	NS
	No	24	55		32	47	
Recurrence							
	Yes	48	56	NS	59	45	**0.04**
	No	10	23		10	23	
	n/a		5		2	3	

LVI: Lymphovascular invasion; PNI: Perineural invasion; NS: Not significant; n/a information not available.

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*Table 3.*Multivariate analysis for the factors influencing overall survival

Variables	Categories	*P* value	HR (hazard ratio) multivariate (95% CI)
Perineural Invasion	No	0.01	1.0 (ref)
	Yes		1.72 (1.10 – 2.70)
T stage	T1-2	0.003	1.0 (ref)
	T3-4		2.22 (1.29 – 3.82)
Extracapsular spread	pN -		1.0 (ref)
	pN +CR-	0.16	1.54 (0.84 – 2.56)
	pN +CR+	0.004	2.99 (1.72 – 5.21)

pN – Negative lymph node; pN+CR- Positive lymph node with no extranodal spread; pN+CR+ Positive lymph node with extracapsular spread

*Table 4.*Multivariate analysis for the factors influencing disease-free survival

Variables	Categories	*P* value	HR (hazard ratio) multivariate (95% CI)
Perineural Invasion	No	0.03	1.0 (ref)
	Yes		1.58 (1.04 – 2.41)
Extracapsular spread	pN -		1.0 (ref)
	pN +CR-	0.15	1.51 (0.86 – 2.65)
	pN +CR+	0.004	2.11 (1.26 – 3.55)

pN – Negative lymph node; pN+CR- Positive lymph node with no extranodal spread; pN+CR+ Positive lymph node with extracapsular spread

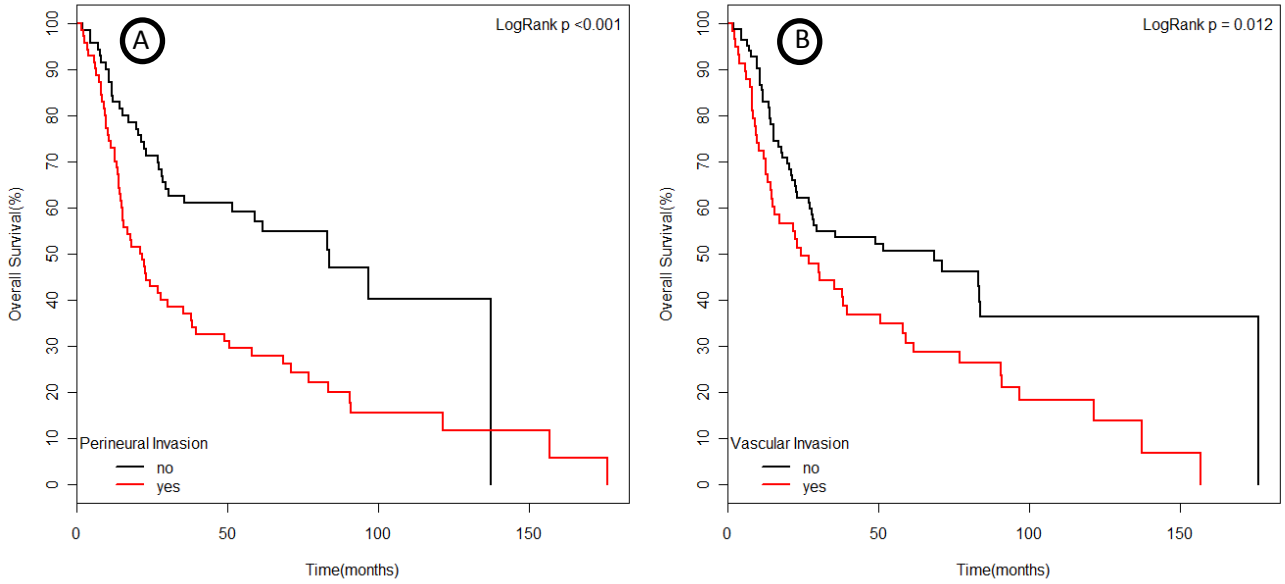

Figure 1. Overall survival in patients with and without PNI (A) and LVI (B)

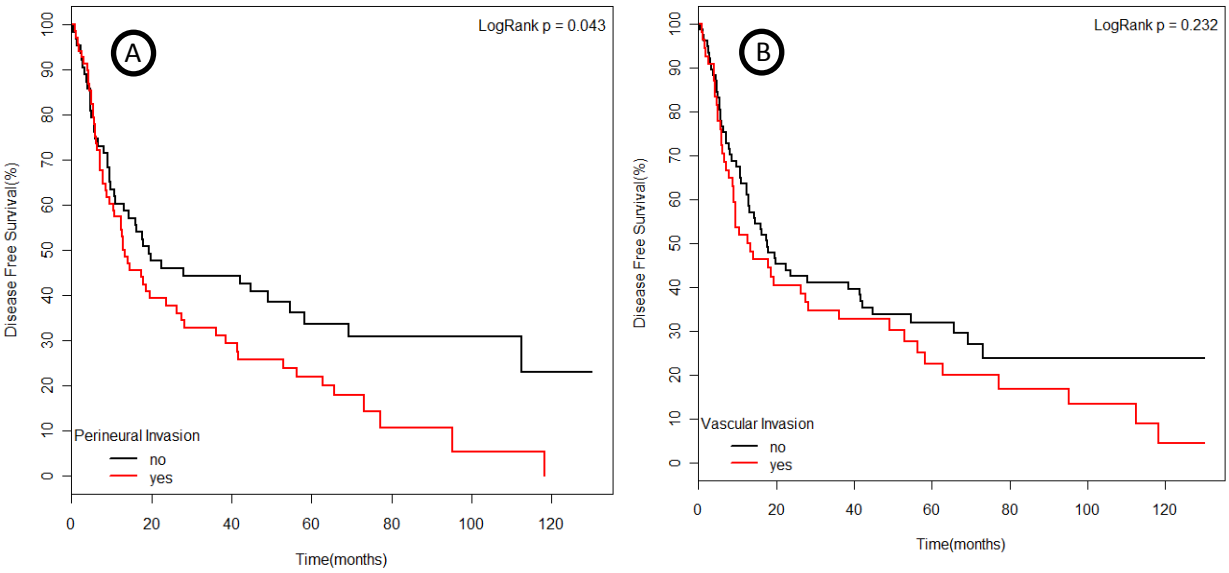

Figure 2. (A) Kaplan–Meier curve for disease free survival according to PNI status and (B) Kaplan–Meier curve for disease free survival according to LVI status

CAPÍTULO 2

Artigo em preparação

EXPRESSION OF MCM-2, CICLYN D1, Ki-67 AND P53 IN ADVANCED STAGE ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is the sixth most common human cancer accounting for over 500,000 new cases annually worldwide. Most

patients had advanced-stage disease at diagnosis and current treatment options frequently incur significant morbidity. Of the known prognostic factors, TNM stage, histologic grade, and tumour thickness are almost universally recognized. However, tumors of the same site with similar histology and stage may behave differently due to their differing biological characteristics. In the search for prognostic factors in oral carcinoma many biomarkers have been used to assess the prognostic value and possible predicting response to treatment. Cell cycle proteins are some of the most tested markers. The aim of this study was to evaluate the expression of Mcm-2, ki-67, Cyclin D1 and p53 and correlate with the prognosis in advanced stage OSCC. Our results demonstrated that increased expression of the Mcm-2 was associated with the presence of extracapsular spread of lymph node metastasis (p= 0.04) and increasing of T stage (p= 0.01). Cyclin D1 was correlated with clinical nodal status (p= 0.02) as well as the expression of p53 (p= 0.02). High expression of Ki-67 was not associated with any of the clinicopathologic parameters analyzed. Overall survival rates in 5 years were associated with the high expression of Cyclin D1 and Mcm-2 and these markers were independent factors to predict survival outcomes. In conclusion the high expression of Mcm-2 and Cyclin D1 have strong impact on survival in advanced stage OSCC.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignancy in the oral cavity with 90% prevalence and represents 34% of the head and neck cancers(1-2). The overall incidence of OSCC varies considerably throughout the world with incidence peaks in Southeast Asia, parts of Central and southwest Europe (Spain, France) and Brazil (2). At present, the most important prognostic factors include histological tumor grade, stage, depth of the tumor invasion, and involvement of regional lymph nodes at the time of diagnosis (1).

However, tumors of the same site with similar histology and stage may behave differently due to their differing biological characteristics and therefore there is an interest in the identification of biomarkers that could be used in the clinical practice in order to better prognosticate, risk-stratify patients and predict

treatment response (3). Among these markers, cell proliferation is regarded as one of the most important biological mechanisms in oncogenesis(4).

Ki-67 is one of such proteins which are expressed in all phases of the cell cycle, except G0, making it an ideal marker for neoplastic tissue (5). Immunohistochemical staining for Ki67 is widely used as a surrogate for proliferation in tumour samples and to analyze its association with prognosis (3). On the other hand TP53 tumour suppressor gene in chromosome 17p13.1 encodes the p53 protein involved in many key events in the cell such as regulation of cell cycle and glucose metabolism in cancer cells, DNA-repair, apoptosis, and senescence, and induced by various stress signals, including DNA-damage and inflammation (3, 6). Aberrations of p53 are the most frequent molecular events in OSCC (6) and most of them are missense mutations that result in an inactivated protein that accumulates in the nucleus, and therefore, it can be detected by immunohistochemistry.

Cyclins plays a key role in cell cycle control and are themselves regulated by cyclin-dependent kinases. Activation of cyclins leads to activation of cell cycle from the G1 phase to the S phase and consequently leads to cell proliferation (7). Cyclin D1 is an oncogene that drives cell cycle progression, and the decision for cell growth or arrest may depend on their concentration (8). Cyclin D1 amplification is one of the most frequent molecular alterations in head and neck squamous cell carcinomas (HNSCC) (9).

The Mcm-2 protein belongs to the family of six minichromosome maintance 2-7 proteins (Mcm 2-7), engaged in recognition and control of DNA replication (10). At the early G1 phase of the cell cycle, MCM proteins are responsible for formation of the pre-replication complex, that differs them from the Ki-67 antigen, which is mostly present in late G1, S, G2 stages of the cell cycle (11). Mcm complexes warrant a replication license and act as helicase unwinding the double helix (10). Some studies suggest that MCM-2 could be used as a biomarker of proliferation and as a diagnostic indicator for many types of cancer including OSCC (10-11).

The purpose of this study was to examine the correlations between the above mentioned markers and clinicopathological features and clinical outcomes in patients with advanced stage OSCC.

METHODS

Paraffin-embedded tissue samples from 88 advanced stage OSCC cases of the oral tongue and floor of the mouth were obtained from the Department of Pathology at AC Camargo Cancer Center, São Paulo, Brazil

All patients were treated at the Department of Head and Neck Surgery and Otorhinolaryngology at the same institution from 1998 to 2009. The eligibility criteria included previously untreated patients, without a second primary tumor and submitted to treatment in the single institution. The National Human Research Ethics Committee approved this study (Protocol # 1684/12). The medical and pathological charts of all patients were examined to obtain clinicopathological data (clinical stage, tumor size, histological grade, lymphovascular embolization, perineural infiltration, pattern of invasion, margins, nodal status), including information regarding lifestyle (smoking habit and alcohol consumption), and demographic data (age, gender, and race).

The tumors were re-staged according to the 2002 version of the International Union Against Cancer (TNM) classification (12). The histological grade was determined on the basis of classification proposed by the World Health Organization (13). Lymphovascular embolization was classified according to the presence or absence of neoplastic cells located in the wall or lumen of blood or lymphatic vessels while perineural infiltration was considered present when the tissue adjacent to the peri and/or intra-tumoral nerves were involved by the neoplastic cells.

Immunohistochemistry

Paraffin-embedded formalin-fixed tissue sections (4 mm) were deparaffinized in xylene and rehydrated in graded ethanol solutions to water. Thereafter, sections were treated with endogenous peroxidase quenching (0.3% H_2O_2 for 15 min) and blocked for unspecific proteins (Novolink Protein Block®, Leica), 20 min each prior to primary antibody incubation. Pressure cooker antigen retrieval consisted of one period at 125°C for 30 min and 90°C for 10 min in 10 mM citric acid solution (pH 6.0) or EDTA/TRIS (pH 9.0) followed by a washing step with

phosphate-buffered saline (PBS). The incubations with the primary antibodies diluted in PBS or ready to use were conducted overnight at 4 °C: details on antigen retrieval methods as well as primary antibody clones, source, and titer are described in *table 1*.

The sections were washed and incubated with secondary antibodies (Novolink™ Post Primary, Leica Biosystems, New Castle, UK) for 30 min followed by the polymer detection system (Novolink™ Polymer, Leica Biosystems) for 30 min at room temperature. Reactions were developed with a solution containing 0.6 mg/ml of 3,30-diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, MO) and 0.01 % H_2O_2 and then counter-stained with Mayer's hematoxylin, dehydrated and mounted with a glass coverslip. Positive controls (a tissue known to contain the antigen under study) were included in all reactions in accordance with manufacturer's protocols.

Immunohistochemical analysis

Immunohistochemical stains were evaluated by automated examination. All slides were digitalized using the Aperio System (Vista, CA, USA), and the resulting images provided were displayed on an LCD monitor with standard contrast, focus, saturation, and white balance. Automated staining intensity was quantified through the Imagescope Software (Aperio System, USA) to compare staining patterns, localization, and intensity between tumors and for each slide at least 1000 cells were counted. The nuclear algorithm was applied in the staining quantitation. The image analysis software provides a cell intensity value as a continuous variable. The image analysis software also provided intensity scores of 1+ to 3+ for individual nuclei, with 1+ being weakly positive to 3+ being strongly positive, but only counting the 2+ and 3+ cells as positive was considered in this study.

The labelling index (LI) was determined as the percentage of markers positive cells among the total number of cells counted in each OSCC specimen and the median value was taken to determine. Therefore the following scores were assigned: "low expression," for value bellow to the LI, and "high expression," for count above the LI of the markers.

Statistical analysis

Analysis of the association between protein levels and the demographic and clinicopathologic characteristics of the patients was performed using the Chi-square test. We analyzed the differences of protein expression between the following different groups: T stage, N stage, Clinical stage, vascular invasion (yes or no), perineural infiltration (yes or no), histologic grade (well differentiated and moderately/poorly differentiated).

Overall and disease-free survival probabilities were calculated based on the Kaplan-Meier method with logrank score for determining statistical significance. Relative risk was evaluated by the multivariate Cox proportional hazards model. The significance level was 5% for all statistical tests. Statistical analyses were performed using using R, version 2.13 (R Development Core Team (2010), Viena, Austria, www.R-project.org).

RESULTS

Clinicopathological data

The study sample consisted of 64 males (72.7%) and 24 females (27.3%) with a mean age of 57 years (range, 27-82 years). The majority of the cases (72.7%) were seen in the tongue. T1 and T2 were presented in 20 (22.7%) cases, whereas T3 and T4 constituted 68 cases (77.3%). The median follow up was of 33.4 months (range 3 to 178 months). The disease free survival was estimated in 30% for 5 years. Overall survival at 5 years was of 38%.

Only 23 cases (26.1%) were node negative and among the positive nodes, 28 (31.8%) cases were N1 and 37 (42.1%) were N2. Most of the cases (59.1%) were TNM stage IV. Among the patients, 43 cases were histologically well differentiated (48.8%), whereas 37 (42.1%) were moderately and 8 (9.1) were poor differentiated. The clinical and pathological parameters of the patients are summarized in *Table 2*.

On univariate analysis to access association of the clinicopathological features with the survival, we obtained that vascular invasion (p= 0.02),

histological grade (p= 0.01), increasing T stage (p= 0.03), lymph node metastasis (p= 0.01), extracapsular spread of lymph node metastasis (p= 0.004) were correlated with overall survival.

Imunohistochemical features

All proteins tested were present in the OSCC samples studied and these markers were stained in the nuclei of neoplastic cells and the controls.

To categorize the samples in positive or negative the value of 10% was used for each marker. Of the p53 cases, 21 were below 10%, and therefore were classified as negative. For cyclin D1, 64 (72%) patients had a 10% or greater of the tumor cells considered positive. Of the Ki-67 cases only 10 cases were considered negative and about the Mcm-2 no one case were below to 10%. For statistical analysis, we grouped the cases according to their LI that were established in 45%, 22%, 23% and 28% for Mcm-2, Ki-67, Cyclin D1 and p53 respectively. In other words, values that were above the LI were represented by a group of tumors that not only had a high proportion positive cells for each marker, but also high intensity of expression.

Our results demonstrated that increased expression of the Mcm-2 was associated with the presence of extracapsular spread of lymph node metastasis (p= 0.04) and increasing of T stage (p= 0.01). Cyclin D1 was correlated with clinical nodal status (p= 0.02) as well as the expression of p53 (p= 0.02). High expression of Ki-67 was not associated with any of the clinicopathologic parameters analyzed.

In further multivariate analysis based on the Cox proportional hazard model, we found that clinical stage (III or IV), and high expressions of mcm-2 and cyclin d1 were independent factor for cancer-specific survival (*table 4*), and overall survial (*table 5*). Yet, Mcm-2 was an independent factor for disease-free survival in the same model of analysis (*table 6*).

DISCUSSION

The TNM classification of oral squamous cell carcinoma is still one of the most important data to categorize the patients and provides a reliable basis for

patient prognosis and therapeutic planning (3). Furthermore, more than 50% of patients presents advanced stage of the disease and it is strong associated with recurrence and poor survival outcomes (1).

In this study, in 88 cases of advanced stage of OSCC, we evaluated the expression of p53, Ki-67 and MCM-2 in OSCC. We have also analyzed the expression of Cyclin D1 and found that there was an association between lymph node metastasis. This finding was also reported in other studies (14).

We also found that higher expression of this marker was associated with a poor prognosis affecting negatively the overall survival. This finding was previous reported by many authorities (9, 14-15).

Carlos de Vicente *et al.* (16) found strong correlation between overexpression of cyclin D1 and regional lymph node metastasis ($p < 0.001$) and advanced stage of tumor ($p< 0.001$). They also reported the relative risk for nodal metastasis in the cases with overexpression was 2.6. In another study Mineta et al. (14) showed that overexpression of Cyclin D1 is associated with poor survival ($p= 0.04$), and progression of lymph node spread in patients with tongue squamous cell carcinomas. Corroborating with this authors our study also reported that cyclin D1 is an idependent factor to cancer-specific survival. Kamingakura *et al*. (17) showed that the gene overexpression of cyclin D1 predicts worses disease-free survival in young as well as old patients, but in the last this marker also affected overall survival.

Cyclin D1 plays a critical role in the transition from the G1 to S phase of the cell cycle (13). Complexes of cyclin D1 and cyclin dependent kinase (CDK) 4 or 6 phosphorylate retinoblastoma (Rb) protein, release E2F transcription factors from Rb protein and consequently, induce the transcription of target genes (17). Protein overexpression and gene amplification of cyclin D1 have been reported in various human tumors, including squamous cell carcinomas of the head and neck (15).

The TP53 tumour suppressor gene in chromosome 17p13.1 encodes the p53 protein that is a nuclear phosphoprotein inhibiting cell cycle progression and inducing apoptosis. It ultimately functions as a tumor suppressor gene. Aberrations of p53 are the most frequent molecular events in human cancers including OSCC. (5). Data from literature show conflicting evidence concerning

the correlation between p53 expression and the most common clinicopathologic parameters in head and neck cancer (18).

In a study performed by Coutinho-Camillo *et al.* (19) p53 had no relation with any clinicopathological parameter. Our study only found a relation between the high expression of p53 and nodal status (p= 0.02).

The Ki67 antibody recognizes a nuclear antigen expressed from mid-G1 to the M-phase of the cell cycle, which thus represents an estimate of the cellular growth fraction (5). However, underestimation of the growth fraction may occur since G1 cells demonstrate weak Ki67 staining and Ki67 rapidly disappears in post-mitotic cells(20).

Our data was showed that Ki-67 was not significantly associated with any of the clinicopathologic parameters analyzed. This fact was in contrary with some authors (4, 16, 19), that have showed an association with nodal metastasis and recurrence.

The Mcm-2 represents a component of the pre-replication complex, and exhibits the activity of helicase, which unwinds the DNA thread during replication (21). In the course of the S-phase, Mcm proteins become irreversibly detached from chromatin, assuring that DNA replication take place only once in the cell cycle (10).

Of interest is the fact that the number of Ki-67 and cyclin D1 positively stained nuclei was always less than that of MCM2-stained nuclei in almost all samples. Similar findings were reported by others authorities (10, 22-23). This fact is explained by the acting of Mcm-2 that is initiated in early phases of the cell cycle and persists throughout it (22).

The high sensitivity and specificity of MCM proteins in distinguishing cycling cells from quiescent cells have prompted a potential clinical application in cancer screening approaches that rely on the detection of malignant and premalignant cells sampled from the surface epithelium (24).

Mcm-2 protein has been confirmed to represent a strong prognostic index in tumors of different locations as breast (11), colorectal (25), esophagus (24), and salivary gland (26).

Kodani *et al.* (23) reported that higher Mcm-2 labeling index might predict malignant transformation of oral dysplasia. Gouvêa *et al.*(27) also reported the

potential of Mcm-2 expression in malignant transformation. The authors studied patients with proliferative verrucous leukoplakia, and found that high immunoexpression of Mcm-2 in mild and moderately dysplasia could be helpful to predict the malignant transformation. In another study Vargas *et al*.(26) showed that MCM-2 was a strong marker of differentiation between benign and malignant salivary gland tumours.

In our study we found that high expression of Mcm-2 correlated with increasing t stage and extracapsular spread of lymph node metastasis and also with poor outcomes in survival, affecting the overall survival and the disease-free survival. This findings were also reported in others studies (10, 22, 28). Moreover we showed that expression of Mcm-2 could be an independent prognostic factor for oral squamous cell carcinoma patients on multivariate analysis, for cancer-specific survival, overall survival and disease-free survival. Similar findings were reported by Szelachowska *et al.* (10) which in their study comparing the effect of Mcm-2 with that of Ki-67 on survival of patients with oral carcinoma, demonstrated higher Mcm-2 estimations. They found that the Mcm-2 proliferation index could serve as an independent prognostic factor for overall survival and disease-specific survival in OSCC and concluded that Mcm-2 expression can be used not only to estimate the proliferative index, but also as a prognostic factor for the survival of patients with oral cancer.

Although biomarkers have been widely studied to better evaluate prognosis in oral cancer (3, 4, 22,29), there is no routine clinical application of them. In fact there is no consensus between the authors about the results on the same marker, and it difficults the universal acceptance of them (29).

In conclusion Mcm-2 is a more sensitive and specific biomarker than Ki-67 and p53 and can be used as good proliferate index. Our results suggest that Mcm-2 and Cyclin D1 protein expressions provide useful information in prognosis and survival in advanced stage OSCC, beyond these marker also represented independents factors to survival outcomes.

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Table 1. Primary serum, clones, source, working titer, and antigen retrieval

Primary Serum	Clone	Source	Working Titer	Antigen Retrieval
Ki-67	MIB-1	DAKO	Ready to use	EDTA/Tris pH 9.0
MCM-2	EPR4120	ABCAM	1:1400	Citrate pH 6.0
Cyclin D1	SP4	DAKO	Ready to use	Citrate pH 6.0
P53	DO-7	DAKO	1:1500	Citrate pH 6.0

Table 2. Summary of clinicopathological features of OSCC patients

Variables	Categories	Patients	%
Age			
	Range	27 – 82	
	Mean	57	
	Median	58	
Gender			
	Male	64	72.3
	Female	24	27.7
Tobacco Smoking			
	Yes	71	80.6
	No	13	14.7
	n/a	4	4.7
Alcohol consumption			
	Yes	62	70.4
	No	18	20.4
	n/a	8	9.2
Tumour Site			
	Tongue	64	72.7
	Floor of the mouth	24	27.3
Tumour Size			
	T1	3	3.4
	T2	17	19.3
	T3	38	43.1
	T4	30	34.2
Nodal Status			
	N0	23	26.1
	N1	28	31.8
	N2	37	42.1
Clinical Stage			
	Stage III	36	40.9
	Stage IV	52	59.1
Histological Grade			
	Well differentiated	43	48.8
	Moderately/poorly differentiated	45	51.2
Recurrence			
	Yes	56	63.7
	No	28	31.8
	n/a	4	4.5
Status			
	Alive	25	28.4
	Dead	61	69.3
	Lost to follow-up	2	2.3

*n/a information not available

Table 3. Association between protein expression and clinicopathological characteristics of OSCC patients

Characteristic	Category	P53 expression		P^a	Mcm-2 expression		P^a	Cyclin D1 expression		P^a	Ki-67 expression		P^a
		Low (%)	High (%)		Low (%)	High (%)		Low (%)	High (%)		Low (%)	High (%)	
T stage	T1/T2	11 (23)	9 (22)	0.87	12 (38)	8 (14)	**0.01**	9 (20)	11 (25)	0.61	4 (16)	16 (25)	0.34
	T3/T4	36 (77)	32 (78)		20 (62)	48 (86)		35 (80)	35 (75)		21 (84)	47 (75)	
N stage	0	18 (38)	5 (12)	**0.02**	7 (22)	16 (29)	0.40	17 (39)	6 (14)	**0.02**	6 (24)	17 (27)	0.57
	1	13 (28)	15 (37)		13 (41)	15 (27)		13 (29)	15 (34)		10 (40)	18 (29)	
	2	16 (34)	21 (51)		12 (37)	25 (45)		14 (32)	23 (52)		9 (36)	28 (44)	
Clinical Stage	III	19 (40)	17 (41)	0.92	16 (50)	20 (36)	0.18	19 (43)	17 (39)	0.66	11 (44)	25 (40)	0.71
	IV	28 (60)	24 (59)		16 (50)	36 (64)		25 (57)	27 (61)		14 (56)	38 (60)	
Histological grade	Well-differentiated	22 (47)	21 (51)	0.67	15 (47)	28 (50)	0.77	22 (50)	21 (48)	0.83	9 (36)	34 (54)	0.12
	Moderately/poorly differentiated	25 (53)	20 (49)		17 (53)	28 (50)		22 (50)	23 (52)		16 (64)	29 (46)	
Vascular invasion	No	28 (60)	25 (61)	0.89	23 (72)	30 (54)	0.09	30 (68)	23 (52)	0.12	17 (68)	36 (57)	0.34
	Yes	19 (40)	16 (39)		9 (28)	26 (46)		14 (32)	21 (48)		8 (32)	27 (43)	
Perineural invasion	No	23 (49)	18 (44)	0.63	18 (56)	23 (41)	0.16	20 (45)	21 (48)	0.81	13 (52)	28 (44)	0.52
	Yes	24 (51)	23 (56)		14 (44)	33 (59)		24 (55)	23 (52)		12 (48)	35 (56)	
Extracapsular spread	No	32 (68)	22 (54)	0.16	24 (75)	30 (54)	**0.04**	31 (68)	23 (52)	0.07	17 (68)	37 (59)	0.42
	Yes	15 (32)	19 (46)		8 (25)	26 (46)		13 (32)	21 (48)		8 (32)	26 (41)	

*$P^{a:}$ *P Value*

Table 4. Multivariate analysis for cancer-specific survival of advanced stage OSCC patients.

Variables	Categories	*P* value	HR (hazard ratio) multivariate (95% CI)
Stage	III	0.013	1.0 (ref)
	IV		2.57 (1.21 – 5.44)
Mcm-2	Low	0.015	1.0 (ref)
	High		2.46 (1.19 – 5.50)
Cyclin D1	Low	0.02	1.0 (ref)
	High		2.43 (1.12 – 5.30)

Table 5. Multivariate analysis for overall survival of advanced stage OSCC patients.

Variables	Categories	*P* value	HR (hazard ratio) multivariate (95% CI)
T stage	1/2 3/4	0.004	1.0 (ref) 3.03 (1.40 – 6.53)
Histological grade	Well-differentiated Moderately/poorly differentiated	0.0002	1.0 (ref) 2.89 (1.65 -5.04)
Mcm-2	Low High	0.0005	1.0 (ref) 2.82 (1.58 – 5.06)
Cyclin D1	Low High	0.03	1.0 (ref) 1.85 (1.04 – 3.28)

Table 6. Multivariate analysis for disease-free survival of advanced stage OSCC patients.

Variables	Categories	*P* value	HR (hazard ratio) multivariate (95% CI)
N stage	0		1.0 (ref)
	1	0.66	1.19 (0.55 – 2.57)
	2	0.03	2.12 (1.06 – 4.24)
Mcm-2	Low	0.01	1.0 (ref)
	High		2.25 (1.24 – 4.11)

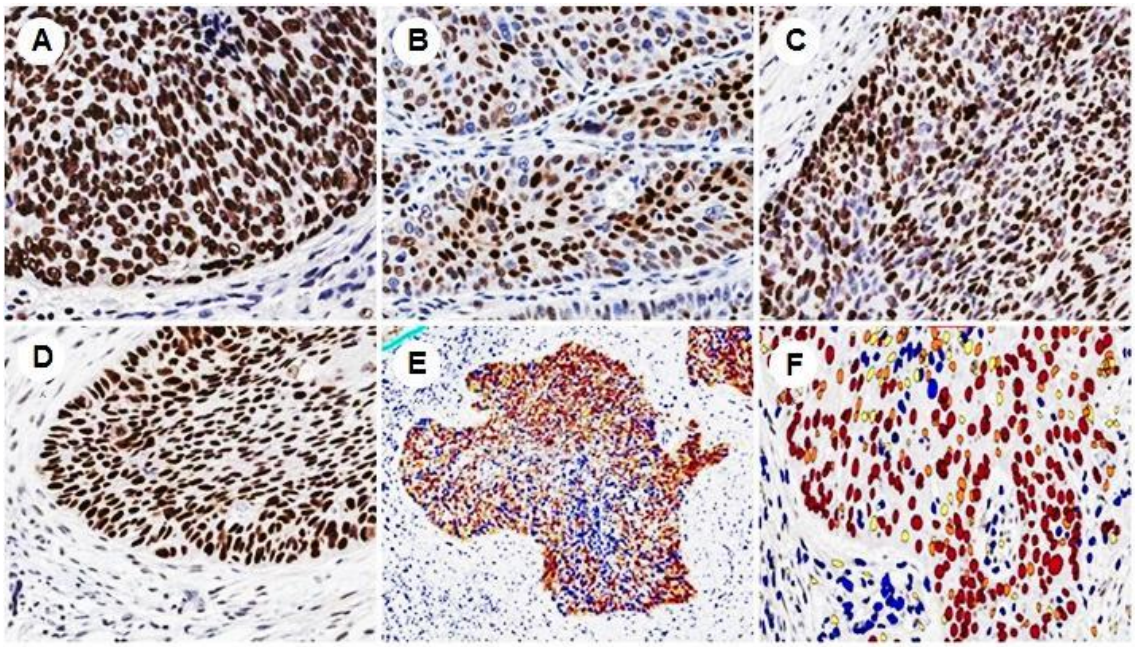

Fig. 1 Immunohistochemistry for Mcm-2 (A), Cyclin D1 (B), Ki-67 (C) and P53 (D) with showing high expression (magnification x400). E and F, Aperio Image Analysis Toolbox results showing variable nuclear colors corresponding to negative (blue), low (yellow), moderate (orange), and high (red) intensity nuclear staining (magnification x200 and x400 respectively).

CONCLUSÃO

Em conclusão, o CEC de língua e assoalho em estádio clínico avançado acomete principalmente homens acima de 55 anos. Fatores como tabaco e bebidas alcólicas estão fortemente associadas à doença. O espécime cirúrgico histopatológico fornece importantes indícios prognósticos, dentre os quais a invasão perineural está relacionada com risco de recorrência e baixas taxas de sobrevida. Encontramos que biomarcadores como Mcm-2 e ciclina D1 são bons preditores de sobrevida, uma vez que altas taxas de expressão estão relacionadas com pobres taxas de sobrevivência. Não se evidenciou associação com o indíce de ki-67, embora este seja considerado um bom preditor de proliferação. Alta expressão de p53 teve correlação com o risco de metástases linfonodais.

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* De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Committee of Medical Journal Editor – Grupo Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

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ANEXO 1

Hospital A.C.Camargo
Centro de Tratamento, Ensino e Pesquisa em Câncer

Comitê de Ética em Pesquisa - CEP

São Paulo, 15 de Agosto de 2012.

Ao
Dr. Luiz Paulo Kowalski

Ref.: Projeto de Pesquisa nº. 1684/12
"Análise Clinicopatológica da angiogênese e linfagiogênese como fator prognóstico em carcinomas espinocelulares de boca em estádio clínico avançado III e IV".

Os membros do Comitê de Ética em Pesquisa em Seres Humanos da Fundação Antonio Prudente – Hospital do Câncer - A.C. Camargo/SP, em sua última reunião de 14/08/2012, **aprovaram** a realização do projeto do estudo em referência e tomaram conhecimento dos seguintes documentos:

- Folha de Rosto para Pesquisa Envolvendo Seres Humanos;
- Termo de Compromisso do Pesquisador com as Resoluções do Conselho Nacional de Saúde;
- Termo de Dispensa do Consentimento Livre e Esclarecido;
- Declaração sobre os Dados Coletados, Publicação dos Dados e Propriedade das Informações Geradas;
- Declaração Sobre o Uso e Destino do Material Biológico, Publicação dos Dados e Propriedades das Informações Geradas;
- Orçamento Financeiro Detalhado;
- Declaração de Infraestrutura e Instalações do Departamento de Cirurgia de Cabeça e Pescoço e Otorrinolaringologia;
- Declaração de Ciência e Comprometimento do Departamento de Cirurgia de Cabeça e Pescoço e Otorrinolaringologia;
- Declaração de Ciência e Comprometimento do Departamento de Anatomia Patológica.

Informações a respeito do andamento do referido projeto deverão ser encaminhadas à assistente do CEP dentro de 12 meses.

Atenciosamente,

Dr. Jefferson Luiz Gross
1º Vice-Coordenador do Comitê de Ética em Pesquisa

1/1

Fundação Antonio Prudente – CNPJ/MF N. 60.961.968/0001-06
Rua Prof. Antônio Prudente, 211 – Liberdade – São Paulo, SP – 01509-900
Telefone: (11) 2189-5000
www.accamargo.org.br

A.C.Camargo Cancer Center

Comitê de Ética em Pesquisa - CEP

São Paulo, 23 de setembro de 2013.

Ao
Prof. Dr. Luiz Paulo Kowalski.

Ref.: Projeto de Pesquisa nº. 1684/12
"Análise Clinicopatológica da angiogênese e linfagiogênese como fator prognóstico em carcinomas espinocelulares de boca em estádio clínico avançado III e IV".

Os membros do Comitê de Ética em Pesquisa em Seres Humanos da Fundação Antonio Prudente – Hospital do Câncer - A.C. Camargo/SP, em sua última reunião de 17/09/2013, **tomaram conhecimento** do seguinte documento:

- Inclusão da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como instituição coparticipante do projeto em referência para inclusão do aluno de Mestrado Juscelino de Freitas Jardim da mesma instituição, sob orientação do Prof. Dr. Luiz Paulo Kowalski, em carta datada de 27 de agosto de 2013;
- Relatório de Acompanhamento do estudo em referência, datado de 27 de agosto de 2013.

Atenciosamente,

Dr. Jefferson Luiz Gross
1º Vice-Coordenador do Comitê de Ética em Pesquisa

Rua Professor Antônio Prudente, 211 • Liberdade • São Paulo / SP • CEP 01509-900
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ANEXO 2

Hospital do Câncer AC Camargo – Fundação Antônio Prudente
Departamento de Cirurgia de Cabeça e Pescoço
Dr. Luiz Paulo Kowalski e Juscelino Freitas
CARCINOMA ESPINOCELULAR T3 E T4

1-Registro Hospitalar.....(_____)

2-Idade:_____(anos).....(_____)

3-Sexo: (1)Masculino (2)Feminino.....(____)

4-Grupo étnico: (1)Branco (2)Negro (3)Amarelo (4)Pardo (5)Outro.....(____)

5-Tempo de queixa (meses):.....(_____)

6-Tipo de queixas:

6a)Dor: (0)Não (1)Sim.....(____)

6b)Tumor: (0)Não (1)Sim.....(____)

6c)Outras: (0)Não (1)Sim.....(____)

7-Localização: (1) Língua (2) Soalho bucal .....(____)

8-Lateralidade: (1)Unilateral (2)Bilateral (3)Mediana.....(____)

9-Biópsia prévia: (0)Não (1)Incisional (2)Agulha (3)Outra.....(____)

10-Tipo Histológico:.....(____)

11-Grau CME: (1)Baixo (2)Intermediário (3)Alto.....(____)

13-Maior diâmetro do tumor (cm):.....(_____)

14-Invasão estruturas adjacentes:(0)Não (1)Língua (2) Soalho (3) Gengiva (4) Músculo (5) Pele (6) Outras.....(____) _____

15- Números de Linfonodos comprometidos clinicamente.....(____)

16-Local das recidivas: Ipsilaterais níveis (0) Não (1) I (2) II (3) III (4) IV (5) V .....(____)

Local das recidivas à distância _____

17-Estádio Clínico (TNM):

Critério T: (1)T1 (2)T2 (3)T3 (4)T4a (5)T4b (6)Tx.....(____)

Critério N: (0)N0 (1)N1 (2)N2a (3)N2b (4)N2c (5)N3 (6)Nx.....(____)

Critério M: (0)M0 (1)M1 (2)Mx.....(____)

18-Estadiamento: (1)III (2) IVa (3)IVb (4)IVc.....(____)

19-Metástases à distância ao diagnóstico:.....(____)

(0)M0 (1)Pulmão (2)Osso (3)Fígado (4)Cérebro (5)Outros

20-Data do início do tratamento:.....(____/____/____)

21-Seqüência de tratamento: (0)Não (1)Cirurgia (2)RXT (3)QT.....(____)

22-Tipo de cirurgia: (1) Glossectomia parcial (2) Hemiglossectomia (3) PG (Pelviglossectomia) (4) PGM (Pelviglossomandibulectomia marginal) (5) PGM seccional (6) Comando (7) Glossectomia total (8) Glossectomia total com mandibulectomia .....( )

23-Esvaziamento

cervical:.....(____)

(0)Não (1)SHOuni (2)Radical (3)Radical modificado (4)Outro

24-Complicaçãoes:.....(____)

(0)Não (1) Deiscência/ Necrose do retalho (2)Infecção (3)Seroma (4) hematoma (5)Fístula (6) Outra _____

25-Metástase em linfonodos após avaliação: (0)Não (1)Sim.....(____)

26- Margens: (0) Livres (1) Exíguas (<5mm) (2) Comprometidas .....(____)

27- Invasão vascular: (0) Não (1) Sim .....(____)

28 – Invasão Perineural: (0) Não (1) Sim .....(____)

29 – Número de linfonodos Ipsilaterais: _____

30 – Número de linfonodos Ipsilaterais: _____

31 – Imunoístoquimica: Marcadores _____

32-Data do inicio da RXT:.....(____/____/____)

33 Dose local (cGy):.....(_____)

34 -Dose cervical (cGy):.....(_____)

35-Recidiva: (0)Não (1)Sim.....(____)

36-Se recidiva regional: (1)Ipsilateral (2)Contralateral (3)Bilateral .....(____)

37-Se recidiva à distância: (1)Pulmão (2)Fígado (3)Osso (4)Cérebro (5)Outro.....(____)

38-Data da primeira recidiva:.....(____/____/____)

39-Tratamento da recidiva:.....(____)

(1)Ressecção local (2)Ressecção recidiva cervical (3)RXT (4)Esvaziamento cervical (5)QT (6)Outra .....( )

40- Número do Anátomo patológico:.....(_____)

41-Data da última informação: .....(____/____/____)

42- Situação da última informação: .....(____)

(1)Vivo sem doença (2)Vivo com doença (3)Morto pela doença (4)Morto por outras causas (5)Perdido de vista

ANEXO 3

Figura 1. Imunomarcação para P53 exibindo fraca intensidade (A) 100X e (B) 400X.

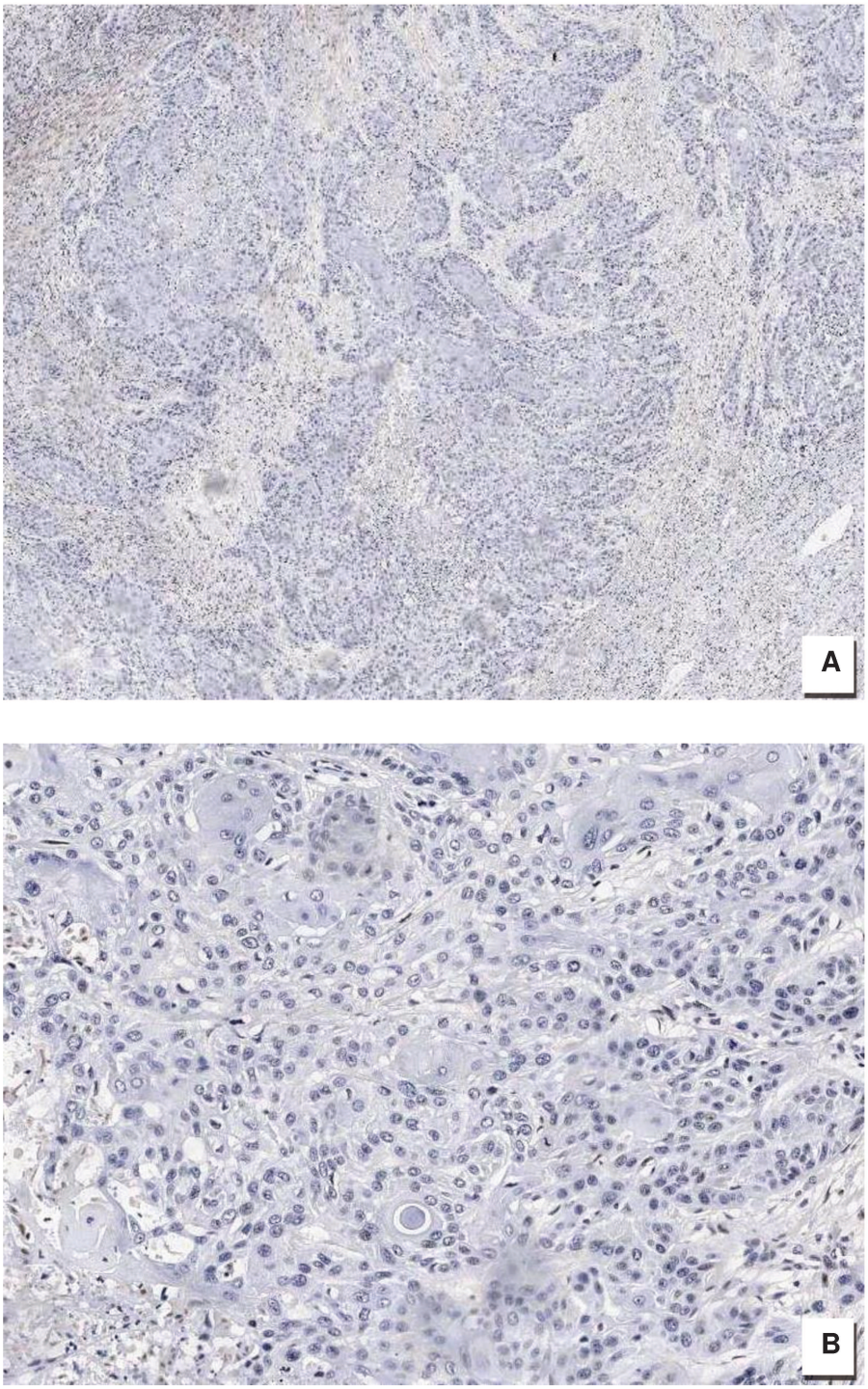


Figura 2. Imunomarcação para P53 exibindo moderada intensidade (A) 100X e (B) 400X.

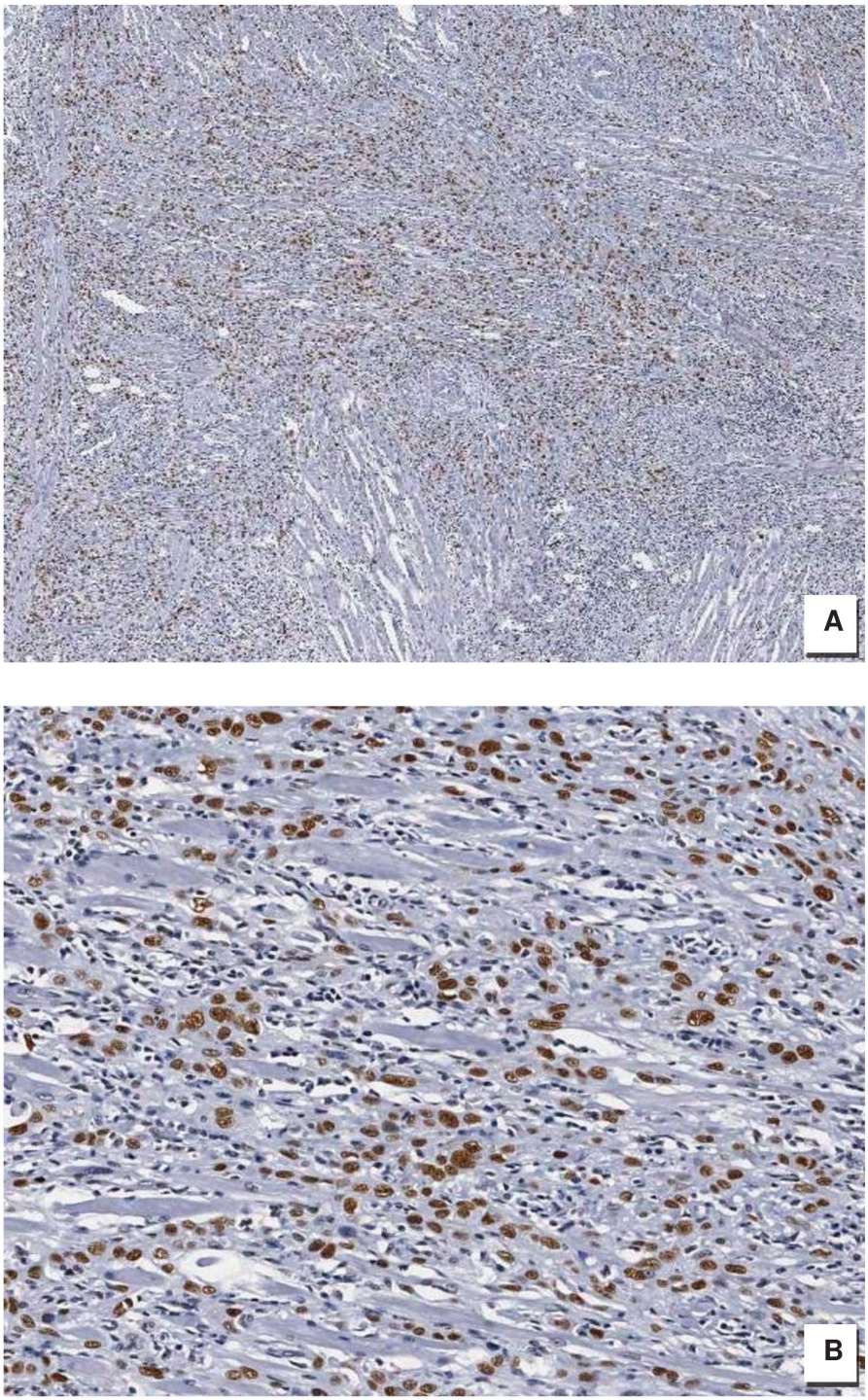
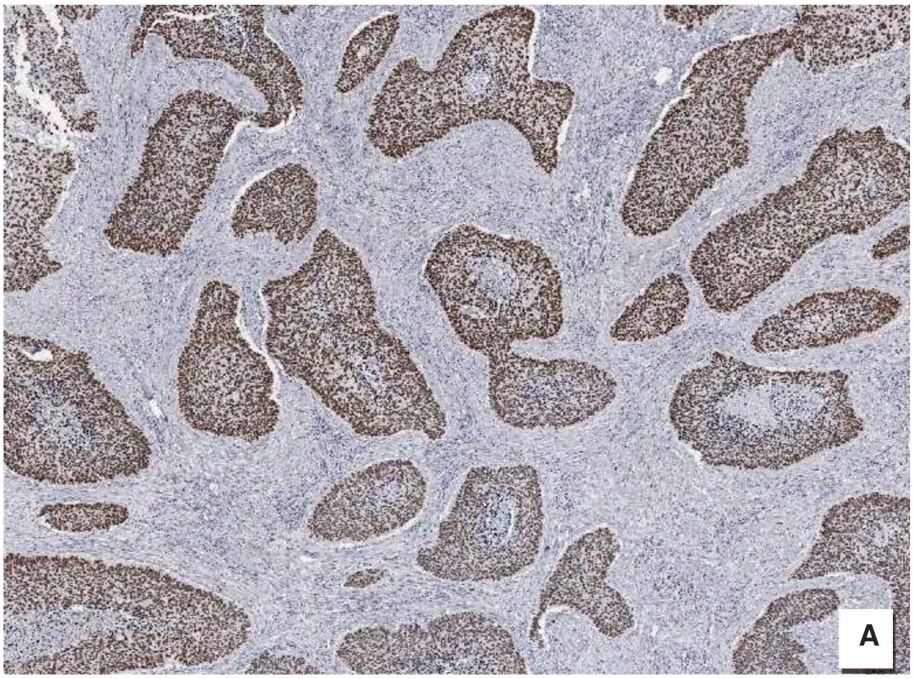


Figura 3. Imunomarcação para P53 exibindo forte intensidade (A) 100X e (B) 400X.



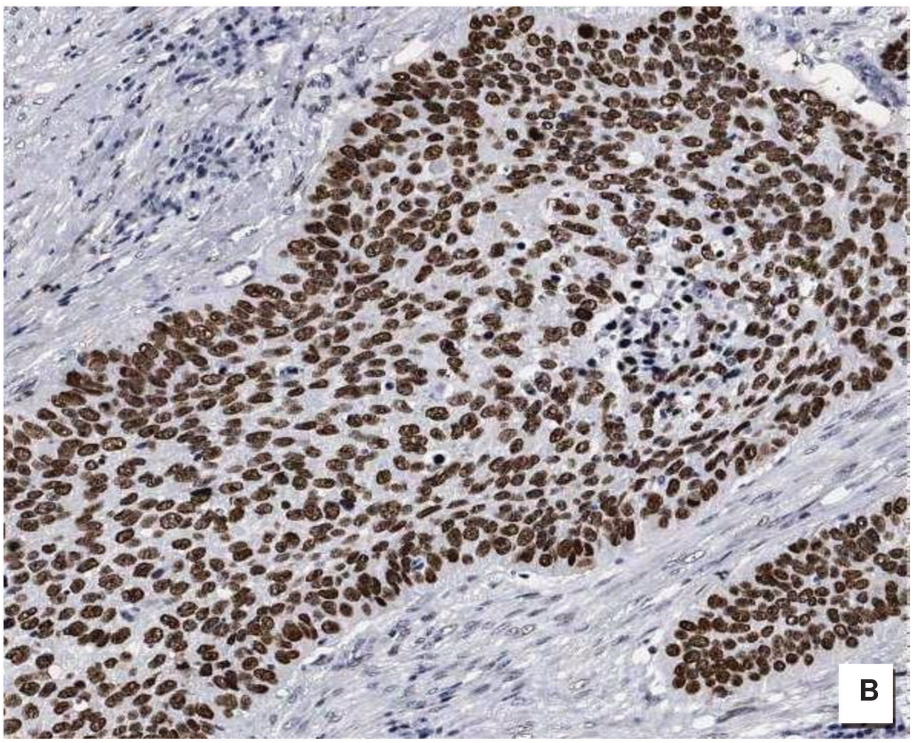


Figura 4. Imunomarcação para Ciclina D1 exibindo fraca intensidade (A) 100X e (B) 400X.

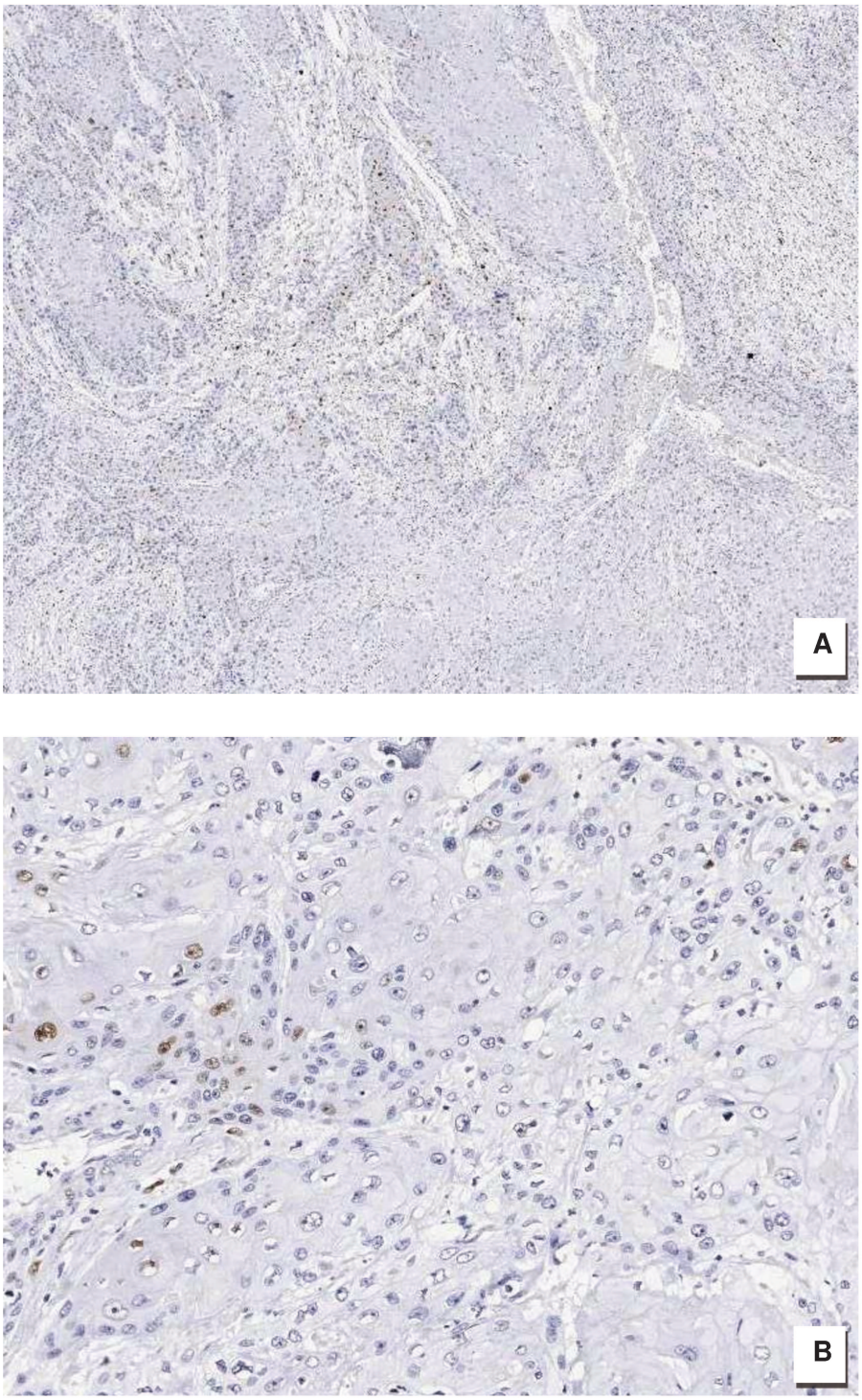


Figura 5. Imunomarcação para Ciclina D1 exibindo moderada intensidade (A) 100X e (B) 400X.

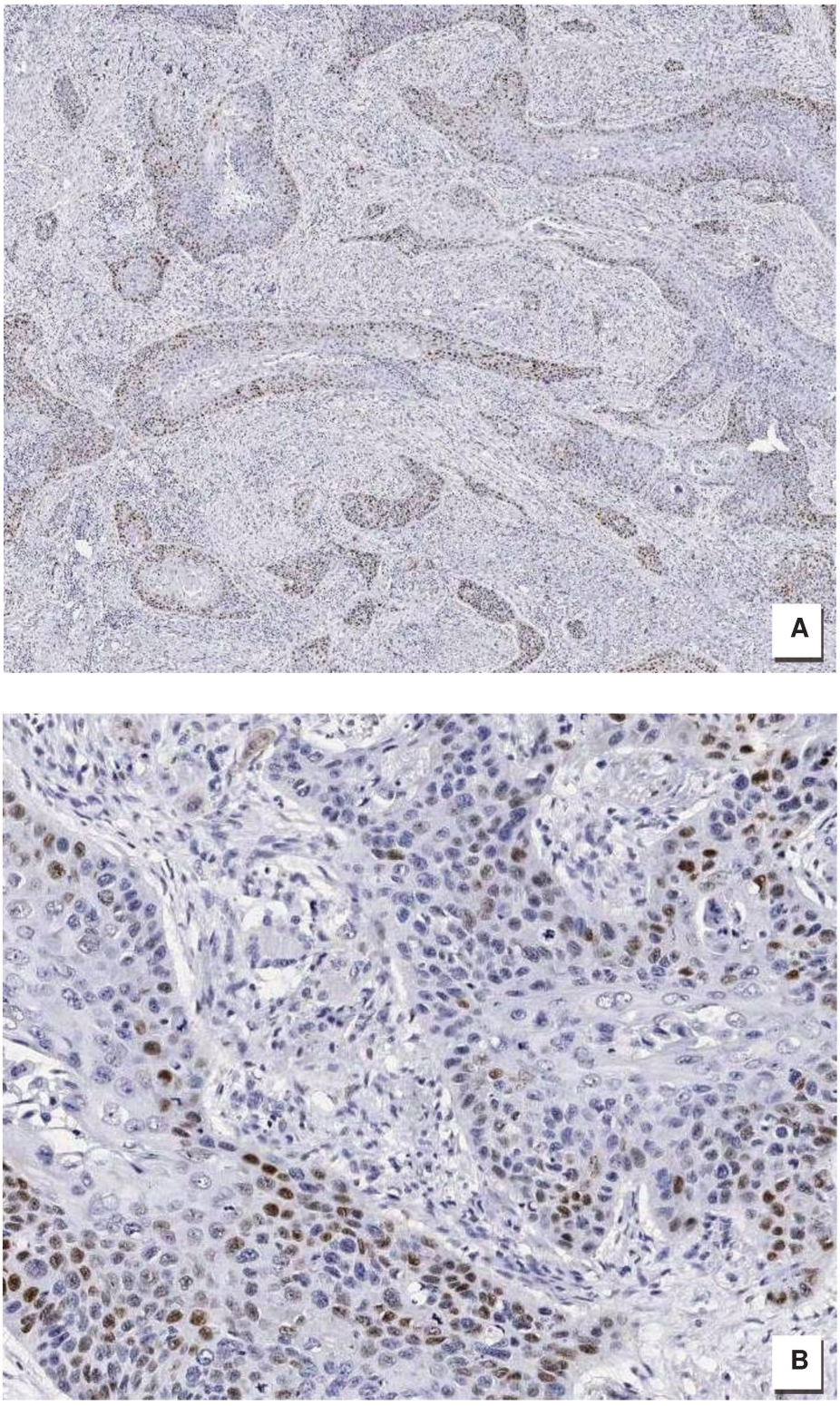


Figura 6. Imunomarcação para Ciclina D1 exibindo forte intensidade (A) 100X e (B) 400X.

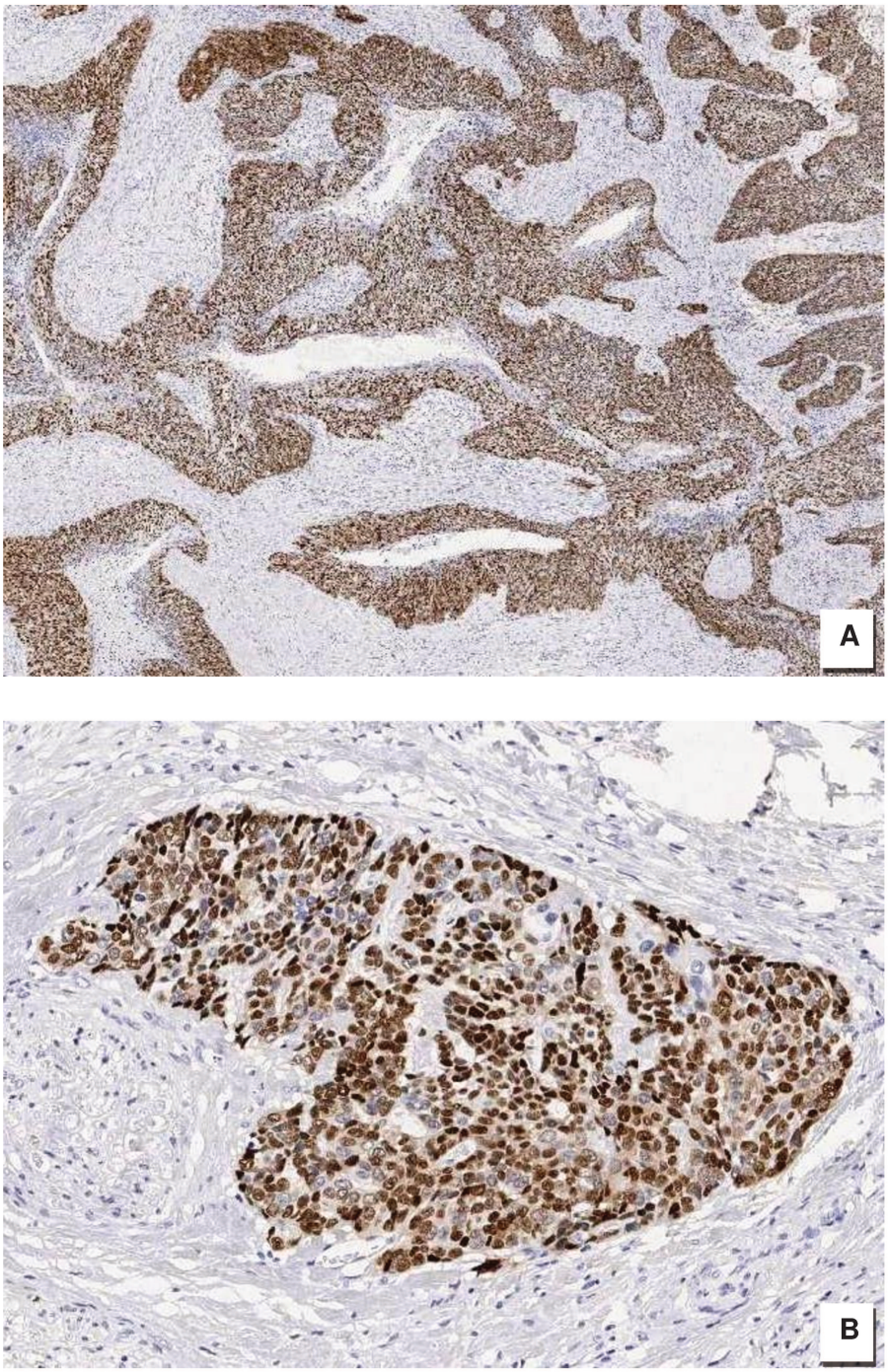


Figura 7. Imunomarcação para Ki-67 exibindo fraca intensidade (A) 100X e (B) 400X.

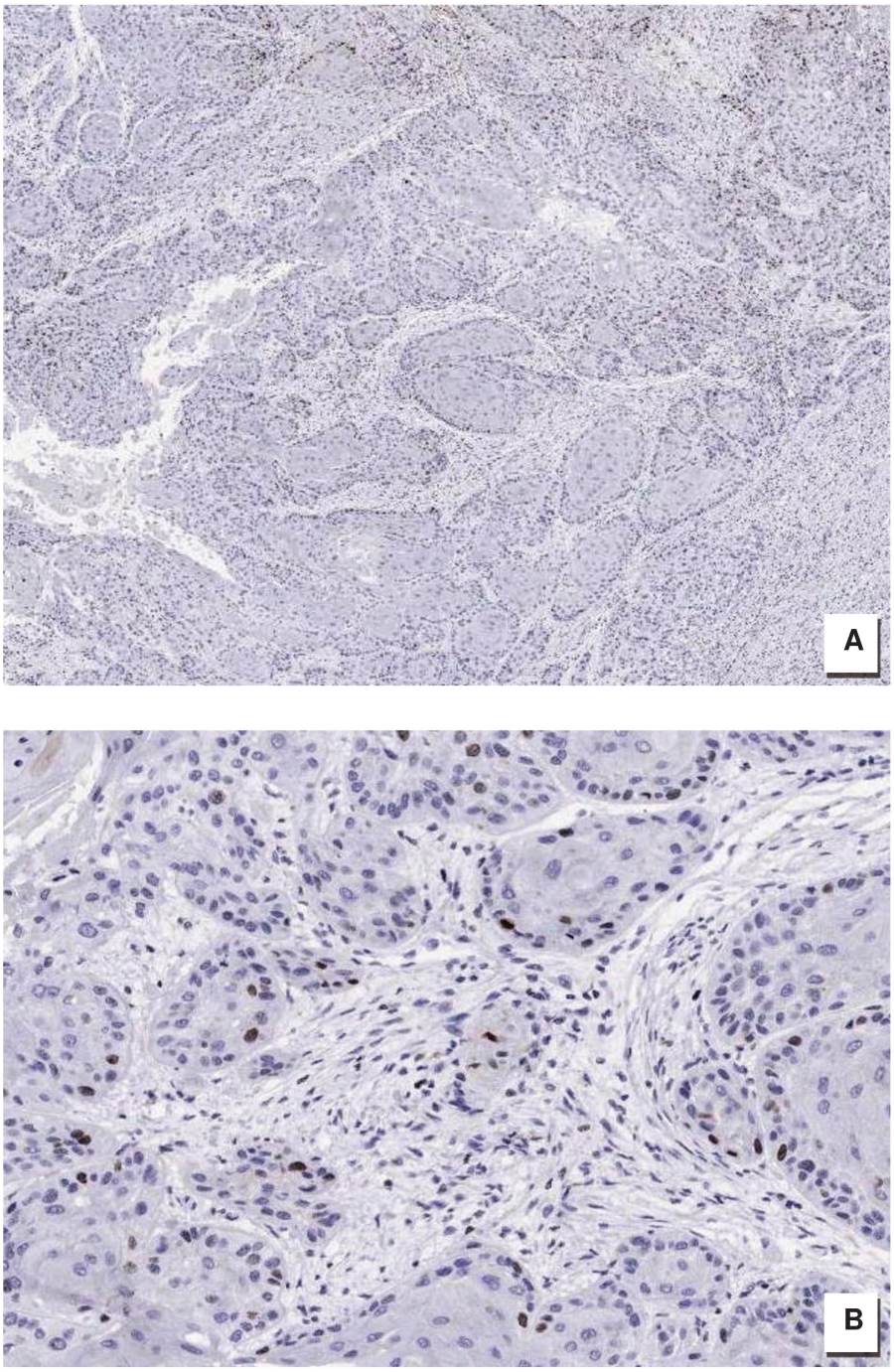


Figura 8. Imunomarcação para Ki-67 exibindo moderada intensidade (A) 100X e (B) 400X.

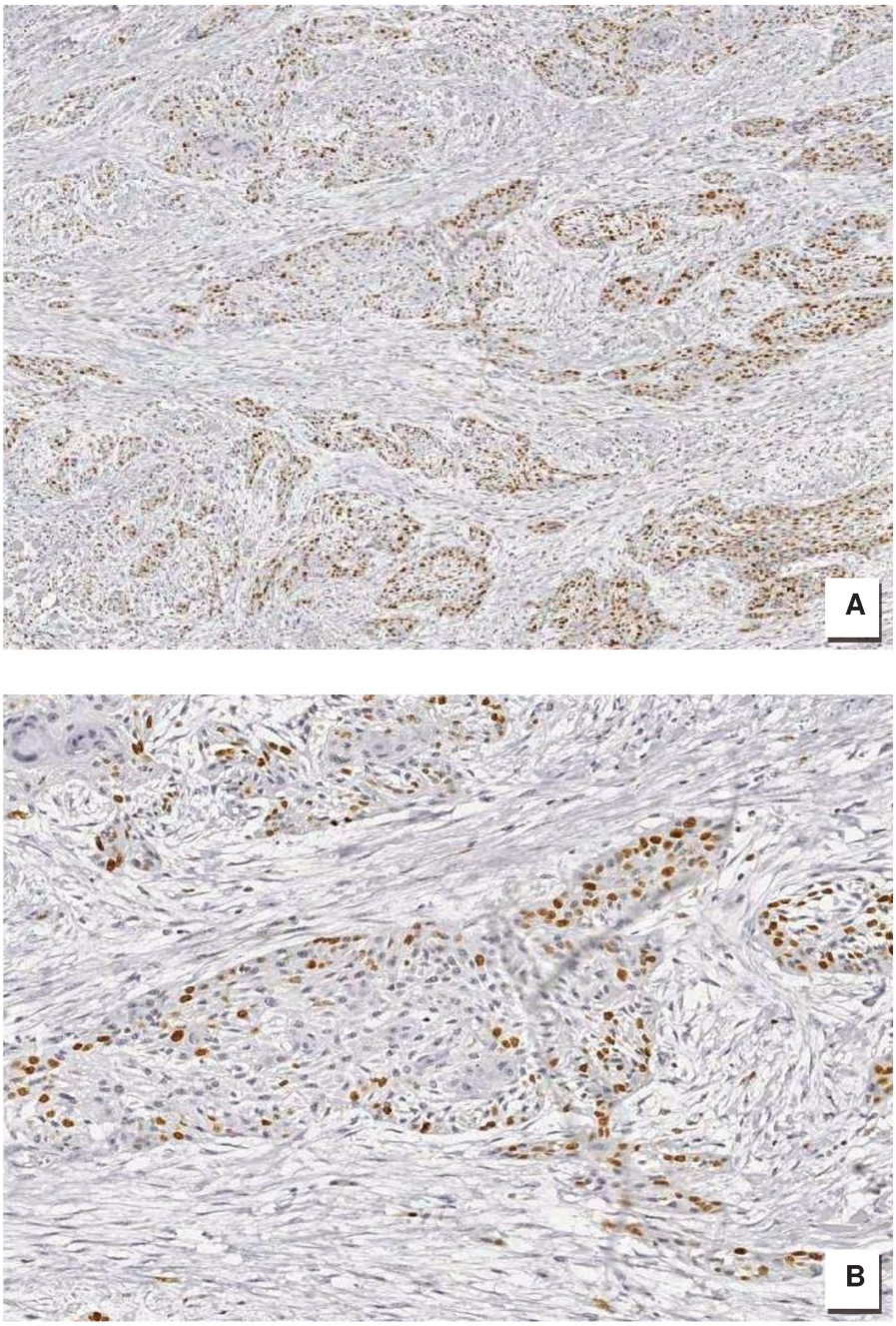


Figura 9. Imunomarcação para Ki-67 exibindo forte intensidade (A) 100X e (B) 400X.

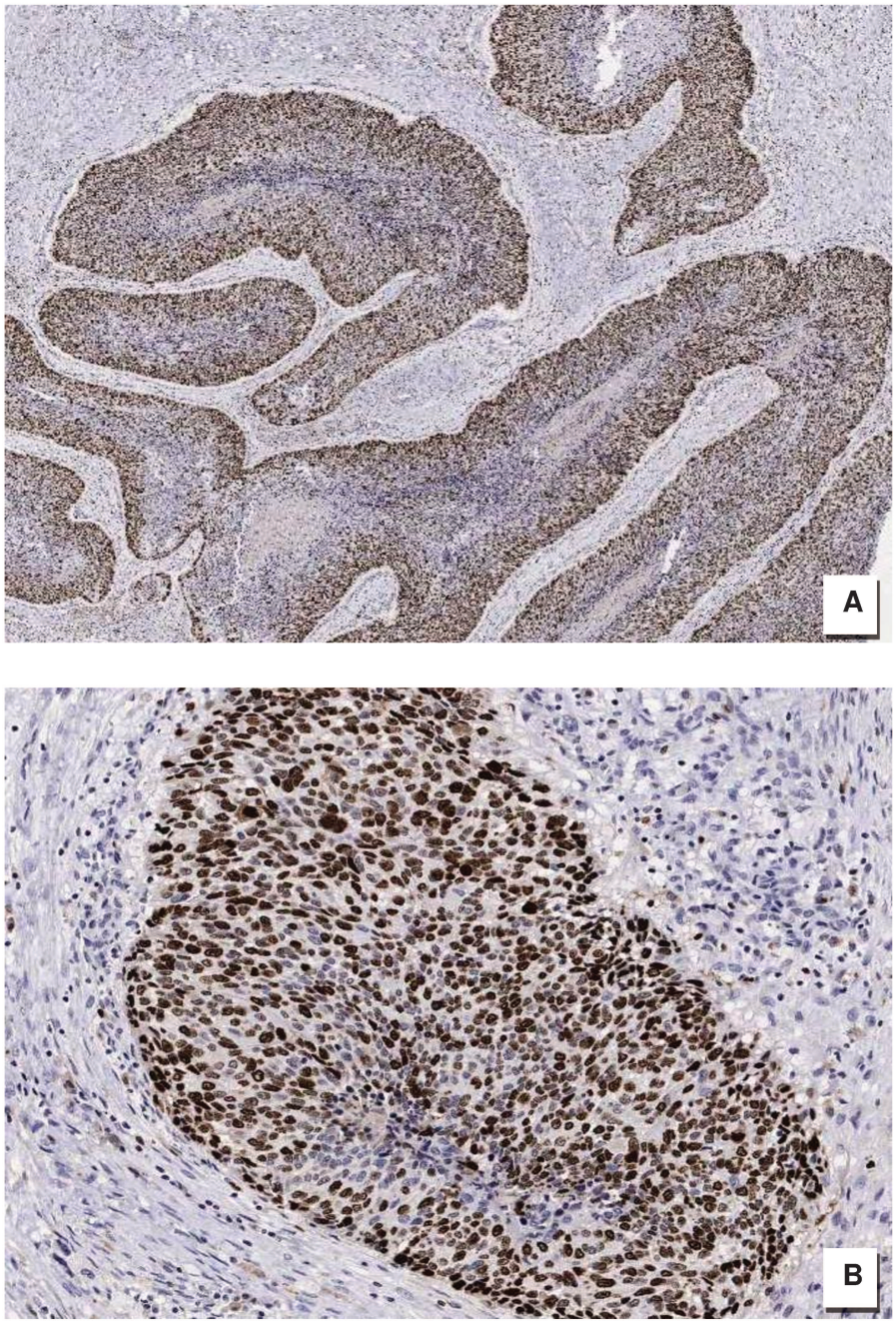


Figura 10. Imunomarcação para Mcm-2 exibindo fraca intensidade (A) 100X e (B) 400X.

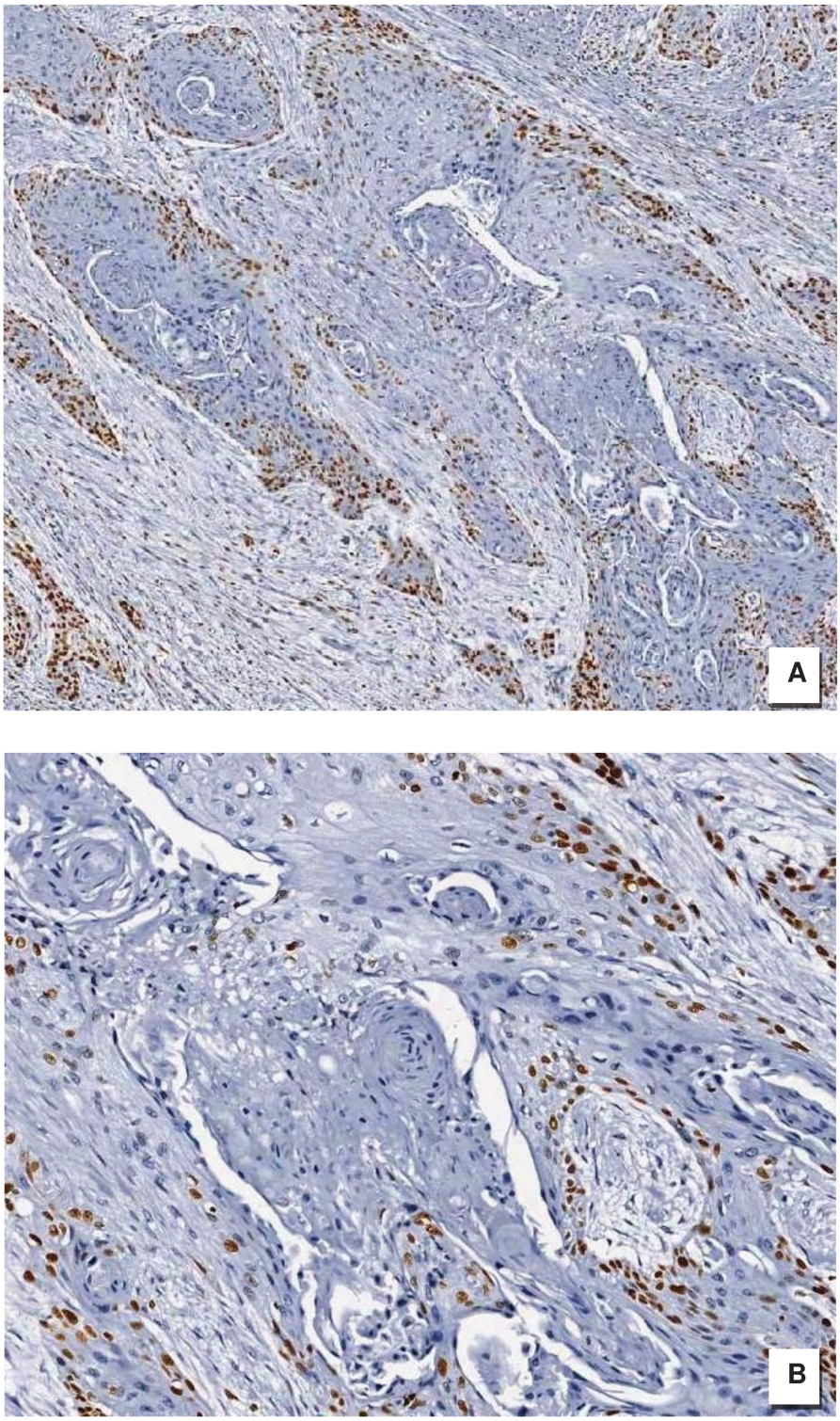


Figura 11. Imunomarcação para Mcm-2 exibindo moderada intensidade (A) 100X e (B) 400X.

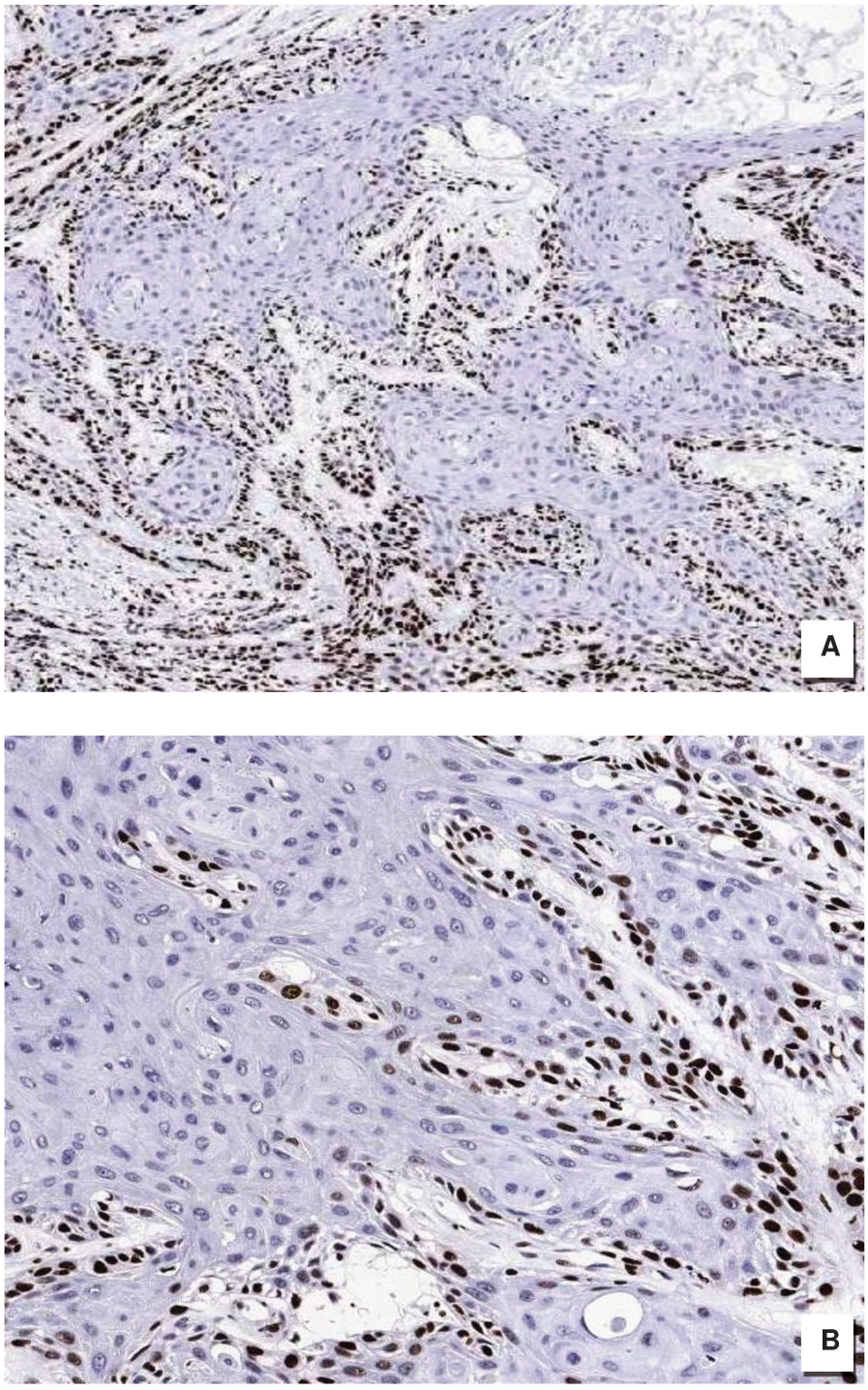


Figura 12. Imunomarcação para Mcm-2 exibindo forte intensidade (A) 100X e (B) 400X.

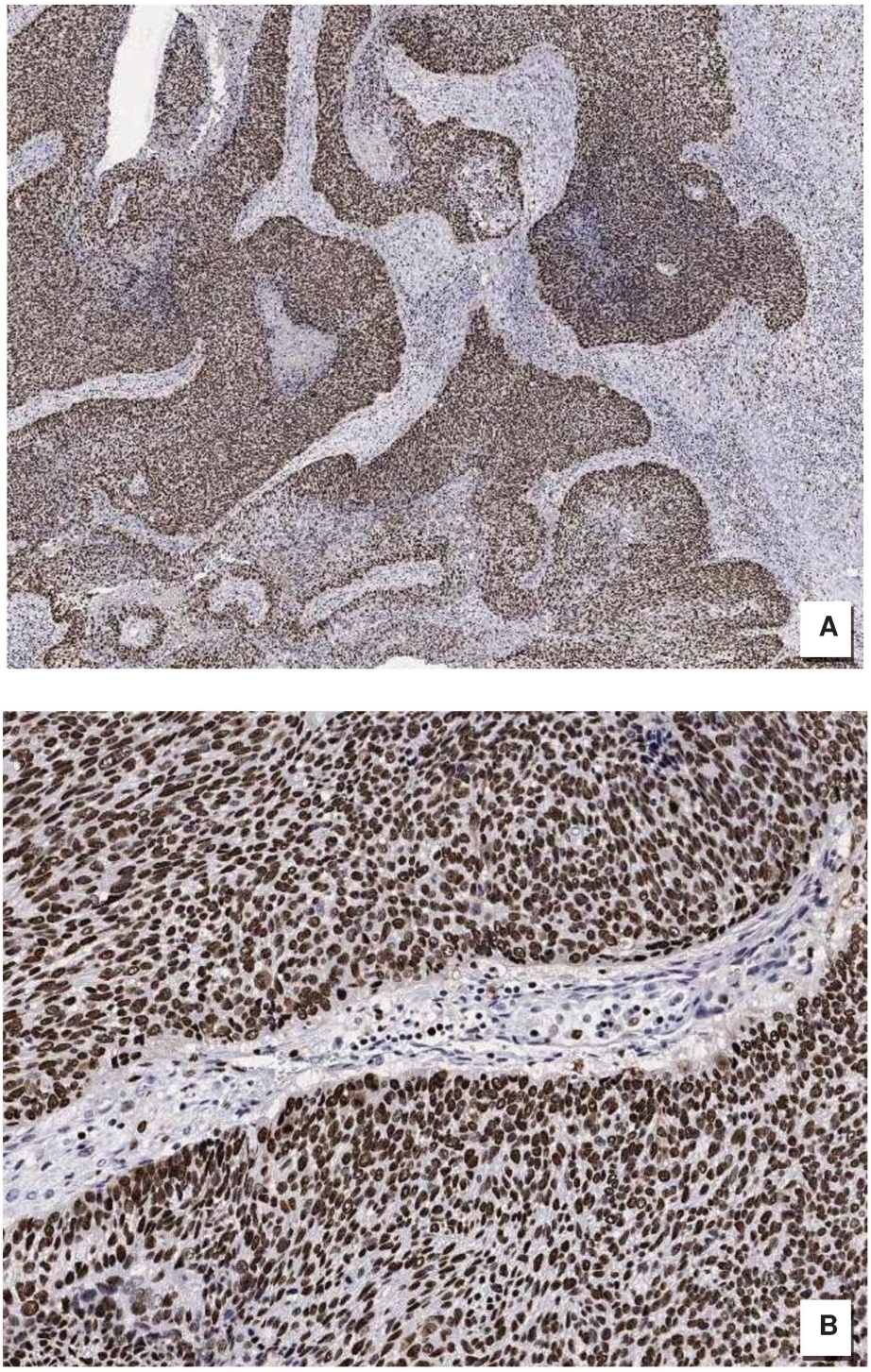


Figura 13. Imagens obtidas do software Aperio Imagescope®, mostrando a metodologia de avaliação de intensidade da marcação. É dado para os scores azul – negativo, amarelo – fraco (+1), laranja – moderado (+2) e vermelho – forte (+3). Aumento de 100x (A) e 400x (B).