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UNIVERSIDADE ESTADUAL DE CAMPINAS
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Orientador: Prof. Dr. Ricardo Della Coletta

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Dissertation of master presented to the Dental School of Piracicaba of the University of Campinas to obtain the master grade in Estomatopathology, area of concentration in Pathology

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PELA ALUNA PRISCILA CAMPIONI RODRIGUES
E ORIENTADA PELO PROF DR RICARDO DELLA COLETTA

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Não há enganos. Os acontecimentos que recaem sobre ti, por muito desagradáveis que sejam, são necessários para que aprendas aquilo que precisas aprender. Cada passo que dás é necessário para chegar ao local que escolheste.

Richard Bach

RESUMO

Embora várias características histopatológicas e moleculares tenham sido propostas como fatores prognósticos do carcinoma espinocelular (CEC) oral, nenhuma ainda é utilizada rotineiramente. Estudos prévios demonstraram que a presença de miofibroblastos no estroma de CECs orais é associada a um pior prognóstico e que pacientes jovens apresentam tumores com comportamento biológico distinto quando comparado ao de pacientes idosos. Os objetivos deste estudo foram 1) avaliar a influência das características demográficas, clínicas e histopatológicas no prognóstico dos CECs de língua, 2) avaliar a frequência de miofibroblastos em displasias orais (leve, moderada e severa), CECs (lesões bem diferenciadas e pobremente diferenciadas) e carcinomas verrucosos (uma variante bem diferenciada do CEC oral) e comparar a frequência destas células com hiperplasias fibrosas (HF) e 3) comparar a densidade de miofibroblastos entre CEC orais de pacientes jovens (<40 anos) e pacientes idosos (>45 anos). Para determinar a influência das características clínicas, demográficas e histopatológicas (risco histológico de Brandwein-Gensler) no prognóstico dos CECs de língua, um estudo retrospectivo com 202 pacientes foi realizado. A detecção de miofibroblastos foi realizada por reações de imuno-histoquímica para a isoforma α da actina de músculo liso (α-SMA) em HFs com epitélio normal (n=29), displasias (n=69), CECs bem diferenciados (n=19), CECs pobremente diferenciados (n=18) e carcinomas verrucosos (n=8). A comparação entre CECs de pacientes jovens e de pacientes idosos foi realizada em um segundo grupo contendo 29 amostras pareadas para localização, estádio clínico e graduação histológica. A análise multivariada de Cox revelou que estádio T, estádio N e recorrência foram fatores independentes das sobrevidas global, específica e livre de doença para os pacientes com CEC de língua. O risco histológico não correlacionou com o prognóstico destes pacientes. HFs e displasias orais não apresentam miofibroblastos, enquanto que 62,2% dos CECs demonstraram miofibroblastos no estroma tumoral. A presença de miofibroblastos foi

significantemente mais frequente nos CECs pobremente diferenciados em comparação aos CECs bem diferenciados ou aos carcinomas verrucosos. Não houve diferença estatisticamente significante entre a densidade de miofibroblastos nos CECs de pacientes jovens e idosos. Os resultados deste estudo demonstram que as características clínicas são melhores fatores preditivos para o prognóstico do CEC de língua do que o risco histológico e que a presença de miofibroblastos não é associada com displasias orais, mas tumores pobremente diferenciados apresentam uma densidade significantemente maior que tumores bem diferenciados. O estudo revelou também que a presença de miofibroblastos no estroma dos CECs de língua não diferencia entre tumores em pacientes jovens e idosos.

Palavras-chave: Carcinoma espinocelular, diferenciação histológica, displasia, carcinoma verrucoso, prognóstico.

ABSTRACT

Although several histopathological and molecular features have been proposed as prognostic factors of the oral squamous cell carcinoma (OSCC), any is routinely used. Previous studies have demonstrated that the presence of myofibroblasts in the stroma of the OSCC is associated with a worse prognosis and that young patients have tumors with a particular biological behavior when compared with older patients. The aims of this study were 1) to evaluate the influence of the demographics, clinical and histopathological features in the prognostic of SCC of tongue, 2) to determine the frequency of myofibroblasts in the oral dysplasias (mild, moderate and severe), OSCC (well differentiated and poorly differentiated) and verrucous carcinoma (a well differentiated variant of the OSCC) and compare the density of this cell with fibrous hyperplasias and 3) to compare the density of myofibroblasts among OSCC of young patients (< 40 years) and older patients (> 45 years). To determine the influence of the clinical, demographic and histopathological (histologic risk of Brandwein-Gensler) features in the prognostic of SCCs of tongue, a retrospective study was realized with 202 patients. Myofibroblasts were detected by immunohistochemical analysis of α smooth muscle actin (α-SMA) in fibrous hyperplasia with normal epithelium (n=29), oral dysplasias (n=69), well differentiated OSCC (n=19), poorly differentiated OSCC (n=18) and verrrucous carcinoma (n=8). The comparison between OSCC affecting young patients and older patients was realized in a second group containing 29 samples paired to localization, clinical stage and histological differentiation. Cox multivariate analysis revealed that the T stage, N stage and recurrence were independent factors of overall survival, disease-especific survival and disease-free survival. The histologic risk was not correlated with the prognostic of the patients. Fibrous hyperplasia and oral dysplasias did not show myofibroblasts in the stroma. The presence of myofibroblasts was higher in the poorly differentiated OSCCs when compared with well differentiated OSCC or with verrucous carcinomas. No significant differences existed between the presence of stromal myofibroblasts of

OSCC affecting young and old individuals. The results of this study demonstrated that the clinical features were best predictive factors to the SCC of tongue prognostic than the histologic risk, and the presence of myofibroblasts was not associated with the oral dyspasias. However the poorly differentiated tumors demonstrated a higher expression of myofibroblasts than well differentiated tumors. The study also revealed that the presence of myofibroblasts in the OSCC not show differences among young and older patients.

Keywords: Squamous cell carcinoma, histopathological grading, dysplasia verrucous carcinoma, prognosis.

Sumário

INTRODUÇÃO

O carcinoma espinocelular (CEC), também denominado de carcinoma de células escamosas ou carcinoma epidermóide, é o tumor maligno mais frequente da cavidade oral e está associado à altas taxas de morbidade e mortalidade (COTRIM *et al*., 2001; BARNES *et al*., 2005; SCULY & BAGAN, 2009). No Brasil, o CEC oral representa a quinta e a décima primeira neoplasia maligna mais comum, respectivamente, em homens e mulheres, com uma média 15.000 mil novos casos/ano (Instituto Nacional do Câncer - INCA, 2013).

O CEC oral acomete principalmente indivíduos do sexo masculino, entre a quinta e sexta décadas de vida, tabagistas e etilistas de longa duração (GERVÁSIO *et al.,* 2001; SCULLY & FELIX, 2006; SCULLY & BAGAN, 2009; SANTOS-SILVA *et al.,* 2011). No entanto, tem se observado um aumento no número de adultos jovens (menos de 40 anos) sendo acometidos em todo o mundo (BARNES *et al*., 2005). Como o acometimento de jovens por CEC oral não é habitual, muitos autores tem buscado estudar este grupo de pacientes com o intuito principal de encontrar diferenças quanto aos fatores etiológicos e o comportamento do tumor. Alguns estudos encontraram uma forte associação entre tabagismo e etilismo, os mesmos fatores associados ao CEC de adultos, com CECs de pacientes jovens (BURZYNSKI et al., 1992; FRIEDLANDER et al., 1998; MACKENZIE *et al*., 2000), mas vários outros não reportaram tal associação (KURIAKOSE *et al*., 1992; SIEGELMANN-DANIELI *et al*., 1998; LLEWELLYN et al., 2004), sugerindo que outros fatores como infecção pelo vírus do papiloma humano (HPV), fatores ocupacionais e deficiência imunológica possam estar associados (ATULA et al., 1996; MACKENZIE et al., 2000; RITCHIE et al., 2003; KAMINAGAKURA et al., 2012; TÚRI *et al*., 2013). Contudo, os resultados são controversos e os fatores etiológicos envolvidos em seu aparecimento permanecem obscuros (RIBEIRO *et al*., 2009; TÚRI *et al*., 2013). Outro ponto ainda controverso é quanto ao comportamento do tumor, uma vez que diferentes autores relataram um curso mais agressivo em comparação ao CEC de adultos

(SARKARIA *et al*., 1994; VARGAS *et al*., 2000; SIRIWARDENA et al., 2007; VERED et al., 2009), enquanto outros autores não encontraram tal diferença (ANNERTZ *et al*., 2002; SASAKI *et al*., 2005; PONTES *et al*., 2011; UDEABOR *et al*., 2012).

Embora a apresentação clínica dos CECs orais seja variável, o aspecto mais comumente encontrado no momento do diagnóstico é o de uma lesão ulcerada presente na cavidade bucal por um longo período de tempo. Outras apresentações são caracterizadas por um aspecto leucoplásico, eritoplásico, eritro-leucoplásico ou exofítico (NEVILLE *et al.,* 2009). O CEC oral pode se originar por meio de uma série de alterações histopatológicas caracterizadas por displasias epiteliais e carcinoma *in situ*, mas devido a várias características, incluindo ausência de dor nos estágios iniciais, o tumor normalmente é diagnosticado em estágios avançados, onde o padrão de lesão ulcerada já é evidente (SEOANE-ROMERO *et al*., 2012). O CEC oral pode se desenvolver a partir de lesões precursoras da mucosa oral (lesões potencialmente malignas), que consistem principalmente de manchas brancas (leucoplasia) ou vermelhas (eritroplasia). A taxa de transformação maligna é superior a 15% para as leucoplasias e quase 100% para as eritoplasias (VERED *et al*., 2007). Alguns fatores como tabagismo e consumo de bebidas alcoólicas, localização da lesão e apresentação clínica estão associados ao risco de transformação das lesões malignizáveis orais, contudo presença de displasia epitelial tem se mostrado como o principal fator (REIBEL, 2003).

As displasias epiteliais são categorizadas em leve, moderada e intensa de acordo com o grau de envolvimento epitelial (BARNES *et al*., 2005; SPEIGHT, 2007). A displasia leve apresenta proliferação ou hiperplasia das células das camadas basal e parabasal que não se estendem além do terço inferior do epitélio. A atipia celular, caracterizada pelo pleomorfismo celular ou nuclear, é geralmente leve. Mitoses não são proeminentes e quando presentes são frequentemente localizadas basalmente e são normais. Em geral, as alterações

arquiteturais são mínimas (BARNES *et al*., 2005; SPEIGHT, 2007). A displasia moderada apresenta proliferação de células atípicas se estendendo até o terço médio do epitélio. A atipia celular é mais intensa que na displasia leve e as alterações como hipercromatismo e proeminente pleomorfismo celular e nuclear podem ser observadas. Mitoses atípicas podem estar presentes, mas são usualmente localizadas na camada basal. As alterações arquiteturais podem ser observadas na metade inferior do epitélio e são caracterizadas por perda de polaridade e hiperplasia, levando a um padrão de cristas epiteliais bulbosas. No entanto, a estratificação e maturação são relativamente normais (BARNES *et al*., 2005; SPEIGHT, 2007). Na displasia intensa há uma proliferação anormal a partir da camada basal para o terço superior do epitélio. As alterações citológicas e arquiteturais são proeminentes. A displasia intensa é caracterizada por um marcado pleomorfismos celular e nuclear e nucléolos múltiplos e proeminentes. Mitoses atípicas e corpos apoptóticos podem ser proeminentes. As alterações arquiteturais são intensas, frequentemente com perda completa de estratificação e queratinização intensa, até mesmo com formação de pérolas de queratina. A presença de cristas epiteliais bulbosas é uma particularidade importante para o diagnóstico das displasias intensas (BARNES *et al*., 2005; SPEIGHT, 2007).

O CEC oral apresenta algumas variantes, incluindo o carcinoma verrucoso (CV), carcinoma escamoso papilífero, carcinoma adenoescamoso, carcinoma de células fusiformes e carcinoma basalóide escamoso (PEREIRA *et al*., 2007). CV foi inicialmente descrito por ACKERMAN em 1948 como uma lesão com aspecto vegetante em couve-flor, superfície rugosa, sulcada e coloração branco-acinzentada podendo conter áreas eritroplásicas. Este tumor é caracterizado por baixa agressividade e bom prognóstico, colocando-o no grupo dos CECs bem diferenciados (ACKERMAN, 1948; MCCOY, 1981; BOUQUOT, 1998; KAUGARS *et al*., 1999; YOSHIMURA *et al*., 2001; ALKAN et al., 2010; AGNIHOTRI & AGNIHOTRI, 2012). O tumor apresenta crescimento lento, comportamento clínico indolente e raramente desenvolve metástases (ACKERMAN, 1948; FERLITO et

al., 1998). Os linfonodos cervicais podem se apresentar tenros e aumentados devido ao envolvimento inflamatório, simulando uma metástase tumoral (BOUQUOT,1998). Microscopicamente o CV oral é caracterizado por uma proliferação epitelial exofítica altamente queratinizada preenchendo as fendas e as cristas epiteliais são arredondadas e apresentam pouca atipia celular. A região perilesional apresenta um padrão de invasão compressivo, o que causa uma destruição no tecido conjuntivo adjacente, podendo em fases mais avançadas chegar ao tecido muscular, ósseo ou glandular (RAJENDRAN *et al*., 1989). A etiopatogenia do CV oral não é bem estabelecida, no entanto, estudos têm demostrado forte associação com o consumo de tabaco, álcool e infecção pelo HPV (TORNES *et al*., 1985; OLIVEIRA *et al*., 2006). O tratamento do CV é basicamente cirúrgico e o prognóstico é frequentemente excelente (Alkan et al., 2010).

Mesmo diante dos significativos avanços nos protocolos terapêuticos alcançados nas últimas décadas e no melhor entendimento dos mecanismos biológicos associados ao desenvolvimento dos CECs orais, a sobrevida dos pacientes em 5 anos é de aproximadamente 50% (MUSTAFA *et al*., 2005; XU *et al*., 2013). Então, a busca por fatores que possam caracterizar o comportamento biológico do tumor, servindo de parâmetro para decidir qual é a melhor abordagem terapêutica para o paciente e auxiliando como ferramenta preditiva de prognóstico, é intensa (SHAH et al., 2009; KIM *et al*., 2012). Nesta linha de pensamento, várias características histopatológicas e sistemas de graduação foram propostos como marcadores do comportamento biológico dos CECs orais (BRODERS, 1941; ANNEROTH et al., 1987; BRYNE et al, 1992; MARTINEZ-GIMENO et al., 1995; BARNES et al., 2005; BRANDWEIN-GENSLER et al., 2005). Contudo, até o momento não existe um sistema amplamente aceito pelos pesquisadores e patologistas orais.

O primeiro sistema de graduação histopatológica dos CECs orais foi descrito por Broders em 1920, que foi adotado mais tarde pela Organização

Mundial de Saúde. Este sistema é baseado em 3 características, grau de queratinização, pleomorfismo celular e nuclear e atividade mitótica, para classificar os tumores em bem diferenciados, moderadamente diferenciados e pobremente diferenciados. Em tumores demonstrando diferentes padrões, fato que é extremamente comum, o padrão mais grave é utilizado para classificação final do tumor. Os carcinomas bem diferenciados apresentam frequentemente um padrão sólido, com proporções variadas de células escamosas e basais, evidente queratinização e poucas figuras de mitoses (BARNES et al., 2005). Geralmente estes carcinomas possuem crescimento lento, margens bem definidas sem invasão vascular e com evidente resposta inflamatória mononuclear (principalmente linfócitos e plasmócitos). Os carcinomas moderadamente diferenciados exibem células neoplásicas bem evidentes com acentuado pleomorfismo nuclear, grande número de mitoses, hipercromatismo, nucléolos bem evidentes e poucas pérolas de queratina, embora possa ocorrer queratinização de células individuais. Geralmente são mais invasivos e mostram crescimento mais rápido que os carcinomas bem diferenciados (BARNES et al., 2005). Os carcinomas pobremente diferenciados apresentam pouca ou nenhuma evidência de queratinização, as células neoplásicas mostram alto grau de pleomorfismo e hipercromasia e o crescimento é difuso com as células demonstrando elevado potencial mitótico (BARNES et al., 2005).

O sistema de graduação histológica mais recente foi descrito por Brandwein-Gensler e colaboradores em 2005. Este sistema é baseado em 3 parâmetros, pior padrão de invasão, resposta inflamatória linfocítica e invasão perineural, que junto classificam os pacientes em baixo, intermediário e alto risco para recidiva e sobrevida, conforme o escore recebido. Os escores atribuídos ao padrão de invasão foram baseados no estudo de Bryne et al. (1992), acrescentando o padrão 5 que corresponde a um padrão de infiltração tumoral vastamente disperso com, pelo menos, 1 mm de tecido normal interposto entre células tumorais e o fronte de invasão do tumor. Os autores preconizaram que o

padrão de invasão a ser considerado deve ser o pior padrão encontrado e não o padrão predominante, já que o primeiro foi preditivo de sobrevida e recorrência, enquanto o segundo foi preditivo apenas de sobrevida (Brandwein-Gensler et al., 2005).

A resposta inflamatória linfocítica na interface tumor/hospedeiro apresenta três níveis. O padrão tipo 1 corresponde a uma banda densa e contínua de tecido linfoide, o padrão 2 é atribuído quando o infiltrado linfocítico é moderado e descontínuo e o padrão 3 representa um infiltrado escasso ou, até mesmo, ausente. A classificação da invasão perineural leva em consideração a ausência e a presença e o tamanho do nervo invadido, sendo nervos pequenos (diâmetro < 1 mm) ou grandes (diâmetro ≥ 1 mm). Este sistema de classificação histopatológica foi validando em alguns poucos estudos.

O mesmo grupo publicou outros 2 estudos demonstrado o valor preditivo de recorrência e sobrevida deste sistema (Brandwein-Gensler et al., 2010; Li et al. 2012). Vered et al. (2010), com um grupo de 50 amostras de CEC de língua, demonstraram que o sistema de classificação histológica de Brandwein-Gensler é preditivo de recorrência e Lindenblatt et al. (2012) demonstraram que o sistema é associado com sobrevidas global, específica e livre de doença de pacientes com CEC oral. Por outro lado, um estudo recente não encontrou associação em um grupo de pacientes com CEC de língua em estágio inicial de desenvolvimento (estadios I e II) (Almangush et al., 2013).

Além de parâmetros morfológicos, inúmeros estudos vêm buscando biomarcadores celulares e moleculares, focando em análises de genes/proteínas específicas ou em análises de interação com o microambiente tumoral (NITTA *et al*., 2011;. ALITALO & DETMAR, 2012), que possam contribuir na orientação da melhor opção terapêutica dos pacientes e na predição do seu prognóstico. Durante o processo de invasão dos tecidos adjacentes, as células tumorais são capazes de induzir uma série de alterações caracterizadas por um acúmulo de

células inflamatórias e imunológicas, capilares sanguíneos e linfáticos, componentes da matriz extracelular (MEC), fibroblastos e miofibroblastos, formando o microambiente tumoral (CAT et al., 2006). Existem várias evidências que todos os componentes do estroma tumoral podem influenciar criticamente a carcinogênese e o fenótipo maligno nas múltiplas etapas do desenvolvimento tumoral (KUNZ-SCHUGHART & KNUECHEL, 2002; GALIÈ et al., 2005). Miofibroblastos são células mesenquimais altamente especializadas que adquirem a capacidade de expressar a isoforma α da actina de musculatura lisa (α-SMA) e de sintetizar níveis elevados de colágeno e outros componentes da MEC (HINZ & GABBIANI, 2003). Estas células apresentam características intermediárias entre fibroblastos e células da musculatura lisa (BADID et al., 2000) e são caracterizadas morfologicamente como células alongadas, fusiformes ou estreladas com núcleo regular e central e citoplasma proeminente, rico em microfilamentos de actina (fibras de estresse) e retículo endoplasmático (MICKE & OSTMAN, 2004). Miofibroblastos podem estar conectados uns aos outros ou com outras células por meio de aderências e junções do tipo gap (DARBY et al., 1990; MICKE & OSTMAN, 2004; TANG et al., 1996) e estabelecem contatos com os componentes da MEC por meio de fibronexus, um complexo transmembrânico formado por actina, integrina e fibronectina (EYDEN, 2001; POWELL et al., 2005). Embora α-SMA seja o marcador mais proeminente para os miofibroblastos (DESMOULIÈRE et al., 2004), esta proteína citoplasmática é encontrada também em outros dois tipos celulares: células musculares lisas e células mioepiteliais. A presença de outros marcadores como laminina, desmina, calponina, miosina de músculo liso, caldesmonina e proteína de ativação dos fibroblastos tem sido utilizada para caracterizar os miofibroblastos, mas o padrão de expressão é variável e dependente principalmente da origem, localização e condição patológica (MICKE & OSTMAN, 2004). De Wever e colaboradores (2008) sugeriram alguns critérios mínimos para a caracterização dos miofibroblastos, que incluem a positividade para α-SMA, vimentina e a enzima de maturação do colágeno tipo I prolil-4-hidroxilase e negatividade para citoqueratinas.

Após o primeiro estudo demonstrando que a presença de miofibroblastos em CECs orais, particularmente na região do fronte invasivo, promove um comportamento mais agressivo ao tumor, resultando em menor sobrevida global do pacientes (KELLERMANN et al., 2007), vários estudos tentaram elucidar o papel dos miofibroblastos no desenvolvimento e progressão tumoral. Em geral, estes estudos confirmaram nossos resultados, revelando que a alta densidade de miofibroblastos é um fator preditivo de prognóstico desfavorável (KELLERMANN et al, 2008; KAWASHIRI et al, 2009; VERED et al, 2010; BELLO et al., 2011; MARSH et al, 2011), e demonstraram ainda que os miofibroblastos no estroma dos CECs orais podem influenciar a proliferação e invasão tumoral, resultando em um tumor mais agressivo (SOBRAL et al, 2011; HINSLEY et al, 2012). No estudo de Sobral et al. (2011) foi demonstrado que produtos de síntese dos miofibroblatos são capazes de modular a proliferação e invasão de linhagens celulares de CEC oral. Contudo, apesar do estudo ter verificado que a síntese elevada de ativina A por miofibroblastos é responsável pela indução da proliferação das linhagens celulares de CEC oral, revelando um dos mecanismos pelo qual miofibroblastos induzem tumorigênese, não foi capaz de caracterizar a(s) molécula(s) envolvida(s) com o processo de invasão tumoral. Identificar as proteínas sintetizadas pelos miofibroblastos com efeitos sobre a proliferação e invasão tumoral é crucial para um melhor conhecimento dos eventos biológicos associados à tumorigênese oral e para a descoberta de novos biomarcadores tumorais, possibilitando, por exemplo, na discriminação de pacientes de alto e baixo risco de desenvolverem metástases e permitindo um tratamento mais individualizado.

Portanto, os objetivos do presente estudo se resumiram em: 1) avaliar o valor prognóstico do sistema de graduação histopatológica de Brandwein-Gensler em um grupo com 202 CECs de língua, 2) avaliar a presença de miofibroblastos nas displasias orais (leve, moderada e severa), no CEC oral bem diferenciado e pobremente diferenciado e no carcinoma verrucoso e comparar a abundância destas células com hiperplasias fibrosas (HF) e 3) comparar a densidade de

miofibroblastos entre CEC orais de pacientes jovens (<40 anos) e pacientes idosos (>45 anos).

CAPÍTULO 1

Clinicopathological prognostic factors of oral tongue squamous cell carcinoma: a retrospective study of 202 cases

ABSTRACT

Background and Objective: Although several histopathological parameters and grading systems have been described as predictive of treatment response and outcome of oral squamous cell carcinomas (OSCC), none is universally accepted. A new scoring system, the histological risk model, was recently described as a powerful predictive tool to recurrence and overall survival in OSCC. The aim of this study was to verify the predictive role of histological risk model in a cohort containing 202 patients with OSCC of the tongue (OTSCC).

Design: Demographic and clinical data were collected from medical records, and the tumors were evaluated according to the histological risk model. Statistical analyses were performed using the chi-square test, the Kaplan-Meier method and the Cox regression model.

Results: The histological risk model showed no statistical correlation with demographic and clinical parameters and did not influence the outcome of the OTSCC patients. However, multivariate regression analysis revealed a significant correlation of clinical disease stage and local, regional and distant recurrence with disease outcome.

Conclusion: Despite major efforts to identify new predictive parameters and histological systems, clinical features are still the most reliable prognosticators for patients with OTSCC.

Key words: Oral tongue squamous cell carcinoma, demographic and clinical parameters, histological risk model, prognosis.

INTRODUCTION

Squamous cell carcinoma (SCC) is the most common malignancy of the oral cavity. Its incidence varies worldwide, with India and South and Southeast of Asia showing the highest rates. It is estimated a global incidence of 275,000 new cases per year (1,2), with approximately 14,000 new cases and 5,000 deaths due to disease in Brazil (3).

Tongue is the most common and deadliest site for OSCC (4,5), particularly its oral (mobile) portion (OTSCC) where the tumor tends to show a more aggressive behavior due to the high frequency of regional lymph node metastasis (6,7). The presence of lymph node metastasis is one of the most important prognostic factors for the survival of patients with OTSCC (8,9). Despite modern surgical techniques and new therapeutic strategies, particularly with new chemotherapy drugs, mortality rates of OTSCC continue to be high in most countries, leading to an overall 5-year survival rate below 50% (1,10). This poor prognosis has encouraged studies to search for new parameters to improve the management and prognosis prediction of the oral cancers. Over one century,

different morphological features have been suggested as predictive of the OSCC prognosis, and on the basis of those features, different histological grading systems of malignancy were developed (11-15). In this line, Brandwein-Gensler et al (16) proposed an updated and modified multiparameter system based on the evaluation of surgical specimens using 3 histological parameters: worst pattern of invasion (WPOI), lymphocytic host response (LHR) and perineural invasion (PNI), which showed significant predictive power for recurrence and overall survival in OSCC (16,17). More recently, the same group validated this predictive model for patients with early-stage OSCC and showed that the model is able to predict patients at high risk for locoregional recurrence and worse prognosis (18).

The present study was conducted to evaluate the correlation of histological risk model proposed by Brandwein-Gensler et al (16) with demographic and clinical features and outcome of 202 patients with OTSCC. Herein, we provide evidences that clinical stage of disease and recurrence are more important on prognostication of patients with OTSCC than the histological risk model.

PATIENTS AND METHODS

Patients

This study was conducted with 202 patients with primary OTSCC who were diagnosed and treated at the Department of Head and Neck Surgery and Otorhinolaryngology, A. C. Camargo Cancer Center, São Paulo-SP, Brazil from 1980 to 2007. The inclusion criteria included complete demographic and clinical data, treatment based on radical surgery with or without postoperative

radiotherapy, availability of paraffin-embedded blocks and follow-up of at least 5 years.

Demographic, clinical and histopathological parameters

Demographic and clinical data, including gender, age, ethnicity, habits such as smoking and alcohol consumption, TNM stage, recurrence, presence of a second primary tumor and survival, were obtained from patient's records. The outcomes were categorized as overall survival, time from treatment initiation until death or last follow-up, disease-specific survival, time from treatment initiation until death due to cancer or last known date alive, and disease-free survival, time from treatment initiation until recurrence (local, regional or distant).

Paraffin-embedded blocks of all cases were retrieved and new sections were stained with hematoxylin and eosin. Slides were assessed independently by 3 investigators, who were blinded to demographic and clinical data and outcomes, and any disagreement was settled by discussion. Tumors were scored regarding WPOI, LHR and PNI, and classified according to the histological risk model proposed by Brandwein-Gensler et al (16).

Statistical analysis

Correlations between histopathological risk model and demographic and clinical parameters of the tumors were performed by cross-tabulation and chi-square test. For statistical proposes, low and intermediate risk were lumped together and compared with high risk. Survival curves were constructed based on the Kaplan-

Meier method and compared with the Log-rank test. For univariate and multivariate survival analysis, the Cox proportional hazard model was employed. The level of significance considered was 5% ($p \leq 0.05$).

RESULTS

Out of 202 OTSCC patients, 110 (54.5%) were men, and the age of the patients ranged from 21 to 95 years, with a median of 58 years. Most of the patients were Caucasians (n=180, 89.1%) and reported smoking (152 patients, 75.2%) and drinking alcohol (141 patients, 69.8%). Regarding clinical stage, 115 (61.5%) patients were classified in early-stage (stages I and II) and 72 (38.5%) in advanced-stage (stages III and IV). During follow up, 52 (25.7%) patients developed local recurrence (34 locally only, 10 local and regional, 5 local and distant and 3 local, regional and distant), 39 (19.3%) developed regional recurrence (21 regionally only, 10 local and regional, 5 regional and distant and 3 local, regional and distant), and 18 (8.9%) patients developed distant recurrence (5 distant only, 5 local and distant, 5 regional and distant, and 3 local, regional and distant). The overall survival ranged from 1 to 262 months, with a median of 59 months (mean 80.2 months). Fifty eight patients (28.7%) died due to the tumor.

The histological risk model based in Brandwein-Gensler' score system classified 22 (10.9%) tumors in low risk, 110 (54.5%) in intermediate risk and 70 (34.6%) in high risk. With respect to the 3 parameters individually, WPOI type 1, 2 or 3 was identified in 138 (68.3%) samples, type 4 in 54 (26.8%) samples and type 5was in 10 (4.9%) samples (Fig. 1). For LHR, 30 (14.8%) cases were classified as

type 1 (strong), 110 (54.4%) as type 2 (intermediate) and 62 (30.7%) as type 3 (weak) (Fig. 2). Perineural invasion was detected in only 24 samples, being 21 (10.4%) samples in small nerves (diameter of less than 1 mm) and 3 (1.5%) samples in large nerves (diameter ≥ 1 mm) (Fig. 3). The correlation between histological risk model and demographic and clinical parameters is depicted in Table 1. No significant correlations were observed between the epidemiological and clinical parameters of the OTSCCs and the histological risk model.

Kaplan-Meier curves revealed that clinical stage and local, regional and distant recurrence were significantly associated with outcome of patients with OTSCC. Patients with tumor at advanced-stage or with recurrence had less favorable prognosis than those with at early-stage or no recurrence, respectively. There were no significant correlations between outcome and age, gender, ethnicity, smoking or drinking habit, treatment, extracapsular lymph node invasion, second primary and histological risk model. The adjusted multivariate analysis based in Cox proportion regression confirmed that clinical disease stage and recurrence were significant predictors of overall survival (Table 2), disease-specific survival (Table 3) and disease-free survival (Table 4) of patients with OTSCC.

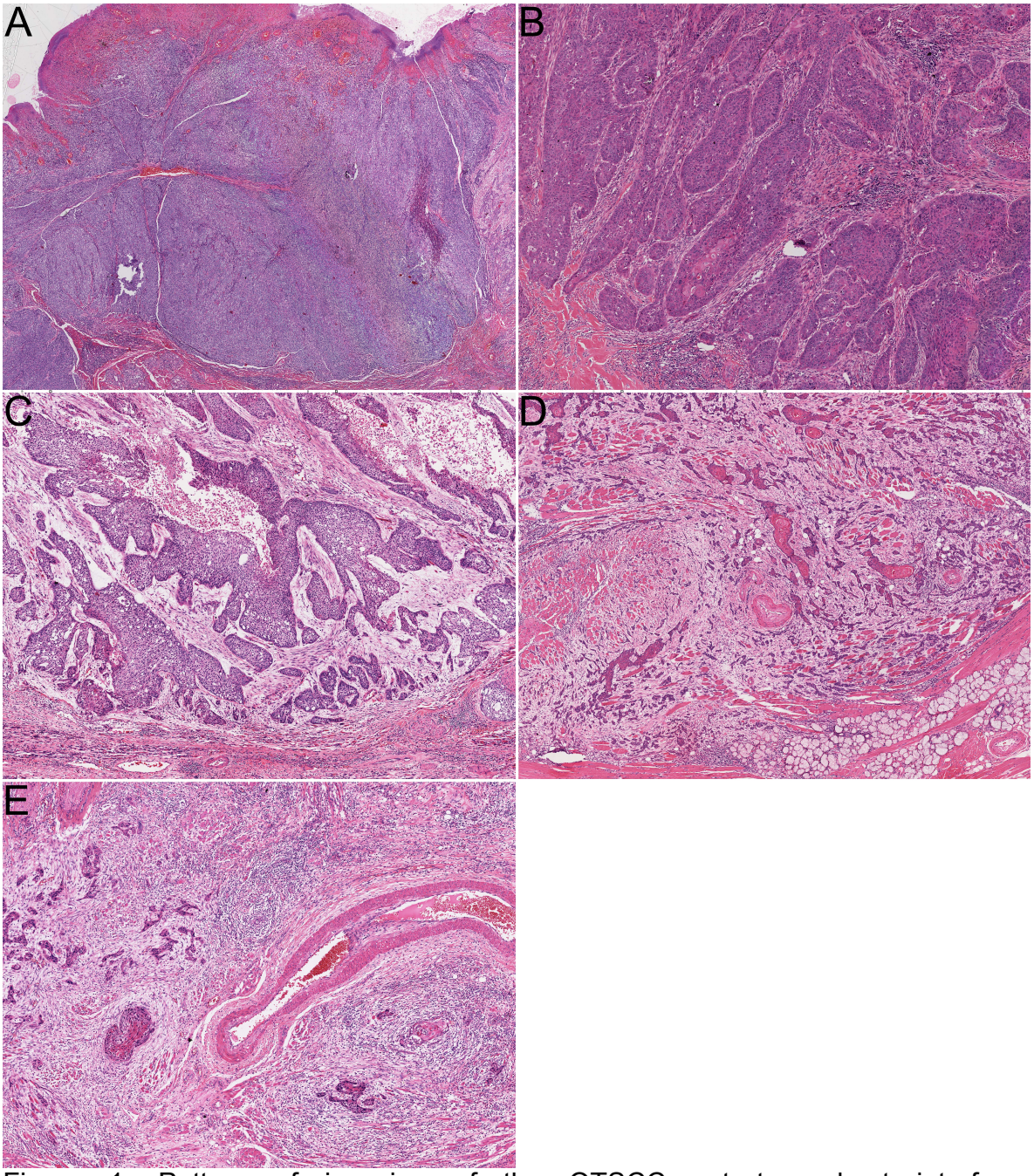

Figure 1. Pattern of invasion of the OTSCCs at tumor-host interface. Representative samples of the 5 types of worst pattern of invasion (WPOI) are showed. (A) Type 1 was characterized by a pushing border pattern of invasion, (B) type 2 represented tumor invasion with broad and separate large islands (finger-like growth), (C) type 3 represented invasive tumor islands with more than 15 cells per island, (D) type 4 was represented by small tumor islands with less than 15 cells per island, and (E) type 5 was characterized by tumor satellites with cells located, at least, 1 mm from the invasive front.

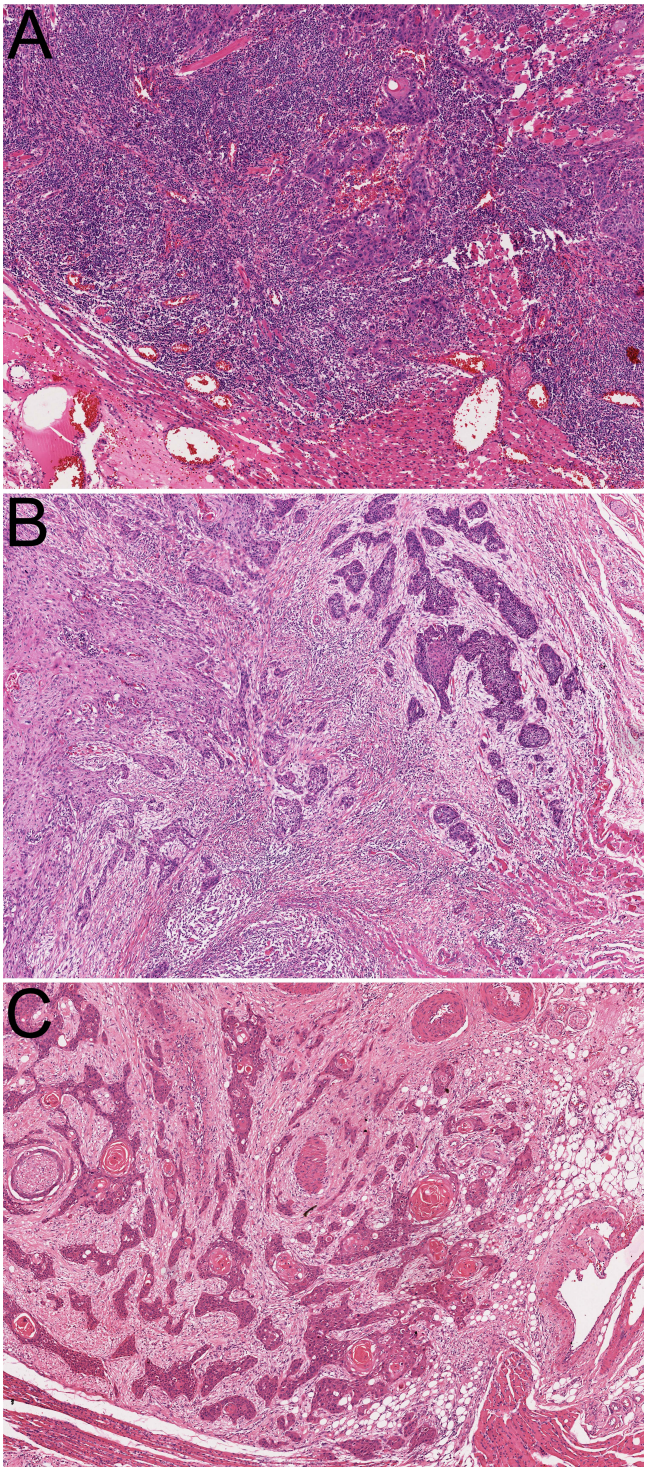

Figure 2. Lymphocytic host response against the tumor. (A) Type 1 showed continuous and dense lymphoid infiltrate at the invasive front interface, (B) type 2 was characterized by discontinuous lymphoid infiltrate and (C) type 3 demonstrated limited response or even lack of lymphoid response.

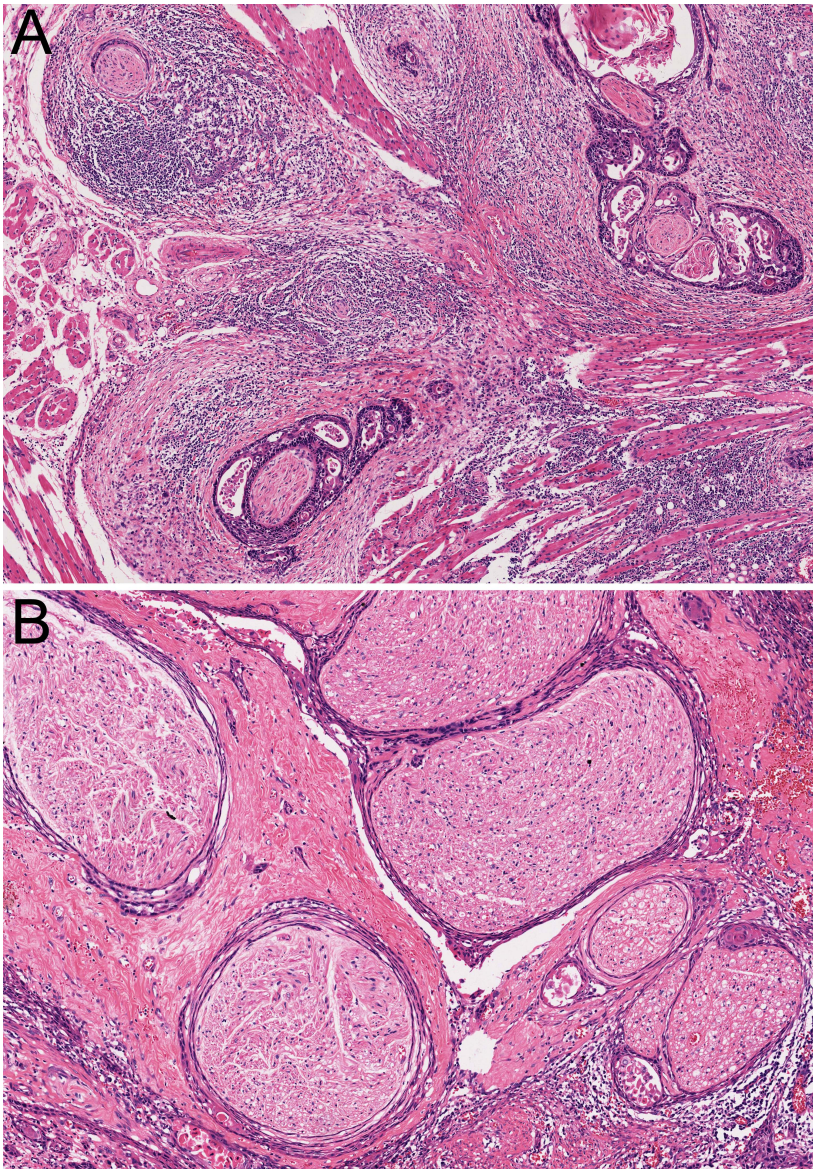

Figure 3. Presence of perineural invasion. Perineural invasion was classified as absent, involving nerves smaller than 1 mm of diameter (A) or nerves larger than 1 mm of diameter (B).

Table 1. Correlation of the demographic and clinical parameters of the OTSCCs with the histological risk model.

Parameter	Risk Model		p value
	Low/Intermediate n (%)	High n (%)	
Age			
< 58	68 (51.5)	41 (58.6)	
≥ 58	64 (48.5)	29 (41.4)	0.33
Gender			
Male	69 (52.3)	41 (58,6)	
Female	63 (47.7)	29 (41,4)	0.39
Ethnicity			
Caucasian	114 (86.4)	66 (94.3)	
Non-Caucasian	18 (13.6)	4 (5.7)	0.10
Smoking habit			
No	31 (24.4)	11 (16.4)	
Yes	96 (75.6)	56 (83.6)	0.19
Drinking habit			
No	41 (32)	13 (19.4)	
Yes	87 (68)	54 (80.6)	0.06
Clinical stage			
Early (I+II)	79 (64.2)	36 (56.3)	0.28
Advanced (III+IV)	44 (35.8)	28 (43.8)	
Extracapsular lymph node invasion			
No	30 (83.3)	17 (70.8)	
Yes	6 (16.7)	7 (29.2)	0.24
Local recurrence			
No	96 (72.7)	54 (77.1)	
Yes	36 (27.3)	16 (22.9)	0.49
Regional Recurrence			
No	108 (81.8)	55 (78.6)	
Yes	24 (18.2)	15 (21.4)	057
Distance Recurrence			
No	121 (91.7)	63 (90)	
Yes	11 (8.3)	7 (10)	0.69
Second Primary			
No	107 (83.6)	51 (73.9)	
Yes	21 (16.4)	18 (26.1)	0.10

Table 2. Cox regression analysis for overall survival of the OTSCC patients.

Parameter	Overall Survival	
	HR (95% CI)/p value	
	Univariate	Multivariate
Age		
< 58	Reference	Reference
≥ 58	0.97 (0.57-1.64)/0.91	0.98 (0.61-1.75)/0.94
Gender		
Male	Reference	Reference
Female	0.62 (0.36-1.05)/0.08	0.71 (0.44-2.72)/0.18
Ethnicity		
Caucasian	Reference	Reference
Non-Caucasian	0.39 (0.23-1.15)/0.11	0.52 (0.36-1.51)/0.15
Smoking habit		
No	Reference	Reference
Yes	1.87 (1.00-3.50)/0.047	1.45 (0.48-1.98)/0.24
Drinking habit		
No	Reference	Reference
Yes	1.67 (0.93-3.02)/0.08	1.29 (0.33-1.71)/0.17
Clinical stage		
Early (I+II)	Reference	Reference
Advanced (III+IV)	4.23 (2.39-7.47)/<0.0001	3.64 (2.40-7.44)/<0.0001
Treatment		
Surgery	Reference	Reference
Surgery + Radiotherapy	1.99 (1.21-3.65)/0.008	1.45 (0.87-3.82)/0.21
Extracapsular lymph node invasion		
No	Reference	Reference
Yes	1.48 (0.63-3.86)/0.33	1.32 (0.25-2.57)/0.52
Local recurrence		
No	Reference	Reference
Yes	6.63 (3.82-11.50)/<0.0001	4.80 (2.41-8.01)/<0.0001
Regional recurrence		
No	Reference	Reference
Yes	14.15 (6.75-29.67)/<0.0001	5.26 (3.56-21.45)/<0.0001
Distance recurrence		
No	Reference	Reference
Yes	22.69 (7.75-66.48)/<0.0001	4.82 (3.15-45.1)/0.0001
Second primary		
No	Reference	Reference
Yes	1.49 (0.76-2.70)/0.27	1.13 (0.45-2.32)/0.64
Histological risk model		
Low/Intermediate	Reference	Reference
High	1.51 (0.89-2.69)/0.12	1.25 (0.47-2.21)/0.20

Table 3. Cox regression analysis for disease-specific survival of the OTSCC patients.

Parameter	Disease-Specific Survival	
	HR (95% CI)/p value	
	Univariate	Multivariate
Age		
< 58	Reference	Reference
≥ 58	1.25 (0.74-2.17)/0.39	1.07 (0.25-2.19)/0.78
Gender		
Male	Reference	Reference
Female	0+90 (0.53-1.53)/0.70	0.94 (0.45-1.88)/0.98
Ethnicity		
Caucasian	Reference	Reference
Non-Caucasian	0.29 (0.22-0.91)/0.027	0.44 (0.19-1.48)/0.08
Smoking habit		
No	Reference	Reference
Yes	1.96 (1.06-3.63)/0.03	1.43 (0.37-1.94)/0.12
Drinking habit		
No	Reference	Reference
Yes	1.82 (1.02-3.24)/0.043	1.20 (0.31-2.97)/0.16
Clinical stage		
Early (I+II)	Reference	Reference
Advanced (III+IV)	2.79 (1.67-4.01)/<0.0001	2.19 (1.16-3.21)/0.001
Treatment		
Surgery	Reference	Reference
Surgery + Radiotherapy	1.83 (1.11-3.29)/0.02	1.54 (0.30-2.89)/0.16
Extracapsular lymph node invasion		
No	Reference	Reference
Yes	1.25 (0.54-3.03)/0.57	1.08 (0.37-1.88)/0.75
Local recurrence		
No	Reference	Reference
Yes	5.70 (3.12-10.41)/<0.0001	4.07 (2.32-8.35)/<0.0001
Regional recurrence		
No	Reference	Reference
Yes	7.49 (3.78-14.85)/<0.0001	5.13 (2.67-12.26)/<0.0001
Distance recurrence		
No	Reference	Reference
Yes	11.78 (4.36-31.77)/<0.0001	3.92 (2.31-22.90)/<0.0001
Second primary		
No	Reference	Reference
Yes	1.09 (0.52-2.31)/0.79	1.02 (0.33-1.90)/0.93
Histological risk model		
Low/Intermediate	Reference	Reference
High	1.63 (0.97-2.97)/0.07	1.48 (0.53-2.02)/0.12

Table 4. Cox regression analysis for disease-free survival of the OTSCC patients.

Parameter	Disease-Free Survival	
	HR (95% CI)/p value	
	Univariate	Multivariate
Age		
< 58	Reference	Reference
≥ 58	1.02 (0.60-1.73)/0.93	1.00 (0.37-1.36)/0.99
Gender		
Male	Reference	
Female	0.57 (0.33-0.96)/0.04	0.82 (0.41-1.78)/0.21
Ethnicity		
Caucasian	Reference	Reference
Non-Caucasian	0.51 (0.23-1.14)/0.10	0.94 (0.87-4.30)/0.22
Smoking habit		
No	Reference	Reference
Yes	1.92 (1.03-3.57)/0.04	1.44 (0.38-1.26)/0.45
Drinking habit		
No	Reference	Reference
Yes	1.74 (0.97-3.12)/0.09	1.42 (0.72-2.02)/0.25
Clinical stage		
Early (I+II)	Reference	Reference
Advanced (III+IV)	4.13 (2.34-7.29)/<0.0001	2.57 (1.94-6.29)/<0.0001
Treatment		
Surgery	Reference	Reference
Surgery + Radiotherapy	1.94 (1.18-3.52)/0.01	1.49 (0.89-2.85)/0.09
Extracapsular lymph node invasion		
No	Reference	Reference
Yes	1.53 (0.66-4.01)/0.29	1.15 (0.24-1.53)/0.65
Local recurrence		
No	Reference	Reference
Yes	14.4 (7.32-28.33)/<0.0001	6.14 (3.53-13.65)/<0.0001
Regional recurrence		
No	Reference	Reference
Yes	59.99 (25.23-142.7)/<0.0001	7.69 (5.80-89.52)/<0.0001
Distance recurrence		
No	Reference	Reference
Yes	54.01 (16.58-175.9)/<0.0001	5.87 (4.89-72.6)/<0.0001
Second primary		
No	Reference	Reference
Yes	1.42 (0.72-2.62)/0.33	1.27 (0.52-2.12)/0.54
Histological risk model		
Low/Intermediate	Reference	Reference
High	1.44 (0.85-2.54)/0.17	1.17 (0.39-1.77)/0.65

DISCUSSION

Major difficulties are encountered to determine the most adequate treatment of OTSCC, which is still mainly based on clinical stage of the tumor, i.e., patients with similar stage are treated in the same way. However, variations in the treatment response and prognosis are high, with some patients presenting prolonged survival while others may die due to regional or distant metastasis shortly (19,20). In the view of those difficulties, major efforts have been made to identify accessible and reproducible parameters that help guide management and consequently prognosis of patients with OTSCC (6,9). In this respect, several authors have proposed that a detailed histopathological staging of the tumors with specific histological systems may have an important role on choice of the best option of treatment and on prognostication of the patients with oral cancer (17,18,21-24). However, the prognostic value of those morphological systems is controversial in the literature (6,25-28). The latest system was proposed by Brandwein-Gensler et al. (16), which evaluates 3 parameters (pattern of invasion, inflammatory response and neural invasion) in surgical specimens. According to the authors, this system represents a simple and highly predictive scoring model of histological risk that can be used for the decision-making about the need for postoperative therapy and prognosis. Since the first report of the histological score model, there are few studies validating it predictive value. The same group has validated this system in 2 independent studies (18,19). In the first one, with a cohort of 305 patients with head and neck carcinomas, the authors showed that patients classified as high-risk showed decreased time to disease progression and lower overall survival (19), and in the

second study the authors demonstrated that patients with early-stage OSCC classified at high-risk have higher risk for locoregional recurrence and shortened disease-specific survival (18). Applying this grading system to 50 cases of tongue SCC, Vered et al. (17) showed that the risk of recurrence was increased for patients classified as high-risk aged less than 60 years. In a cohort with 53 primary OSCC, Lindenblatt et al. (20) showed that the histological risk model was significantly correlated with overall, disease-specific and disease-free survival. On the other hand, a recent study with early-stage OTSCC could not prove the correlation between histological risk model and survival (29). The results of the present study also showed a lack of correlation of histological risk model with epidemiological and clinical features, as well as disease outcomes.

The 3 parameters contemplated in the histological risk model have been individually described as predictive of prognosis for OTSCC patients, but none of them showed high reproducibility. The WPOI at tumor/host interface reflects how the cells invade the adjacent tissues. Behind this feature, there are important phenomena related to tumorigenesis such as epithelial-mesenchymal transition, secretion of degrading enzymes, interactions cell-cell and cell-extracellular matrix and motility of the cells (30,31). Previous studies have demonstrated that tumors formed by small cell islands, either individually or loosely arranged as nests, or forming satellite tumors are more aggressive than tumors with large islands, resulting in worst prognosis (15,16). However, those specific features occur much less commonly, making the distinction of the tumor behavior based on this aspect limited. Furthermore, the pattern classified as type 3 WPOI, which is characterized

by tumor islands containing more than 15 cells, is by far the most common pattern of invasion in OTSCC, as identified in the present cohort.

The most intriguing feature of the histological risk model is the power of the LHR. Tumors with little or none inflammatory infiltrate are classified, independent of any other feature, as high-risk tumors showing consequently high risk for locoregional recurrence and shortened survival. An important dilemma exists regarding the role of the immune and inflammatory responses to tumors, with both antitumoral and protumoral effects described (32). Traditionally, the immune and inflammatory responses were considered as an attempt of organism to eradicate tumor cells, and indeed, to evade immune destruction is considered one of the hallmarks of the cancer (33). However, the immune and inflammatory cells also demonstrate the paradoxical effect of enhancing tumorigenesis (34). In favor of this protumoral effect, immune and inflammatory cells are able to produce several signaling molecules that serve as promoters of tumor growth and expansion, including growth factors such as EGF, VEGF and FGF2, chemokines, cytokines and matrix-degrading enzymes such as matrix metalloproteinases, cysteine cathepsin proteases and heparanase (35,36). Thus, the assumption that high density of lymphocytes is a morphological feature in favor of a low-risk tumor with good prognosis seems an equivocal, because there are clear evidences that the immune and inflammatory cells can invoke both tumor-promoting and tumor-antagonizing effects, depending of the context and of cell types.

Regarding PNI, there are no consistent evidences of its effects on tumor growth and spread, with few studies showing prognostic significance in oral

cancers (37-39). On the other hand, tumor invasion of lymphatic and blood vessels is not considered in the histological risk model, but has been long recognized as independent prognostic factor on several multivariate studies by increasing the risk of lymph node and distant metastases (22,40,41). It is also an important indication of adjuvant therapy for many oncologists (42). Furthermore, vascular infiltration is a more frequent feature identified in OTSCC compared to perineural invasion.

Although the current study did not find correlation with the histological risk model, clinical stage of the disease and recurrence were correlated with survival of OTSCC patients. Tumor size may influence in choice of treatment, particularly the ability of the surgeons to perform complete resection in deep invading tumors, and the outcome of the patients (43). Furthermore, increased tumor size has been associated with regional metastases, high recurrence rates and poor prognosis (22,44,45). Similarly, regional lymph node metastasis, the other feature of the clinical stage, since all patients were classified as M0 at initial diagnosis, is considered the most important prognostic factor to OTSCC (9, 46) and presence of extracapsular spread worsens the prognosis (47). Although poor differentiation was described as a factor that influence tumor spread to lymph nodes via lymphatic vessels (48), no correlation between histological differentiation and N status was observed in this cohort (data not shown). Although an unexpected high number of tumors were classified in early-stage (T1N0M0 or T2N0M0), our findings reinforced that the advanced-stage is an independent marker of patient's outcome. The adjusted multivariate analysis based in Cox proportion regression revealed that patients with early-stage tumors showed a 5-year disease-specific survival of

79.9% compared with 53.1% for patients with advanced tumors. Thus, the current study confirmed that clinical stage is a good indicator of OTSCC prognosis. Recurrence of the disease is a significant cause of death in OTSCC, particularly relapse in the neck which is the most frequent form of recurrence. Several factors may influence the rate of recurrence, but the most important are pathological extension of the primary tumor and of metastatic disease at the time of the diagnosis and type of treatment to control both tumor and its metastasis. In general, the salvage treatment in patients with recurrent disease is difficult and the cure rates is low (49,50). The current study confirmed those aspects, since patients with recurrence (local, regional or distant) had significantly shorted survival rates than patients without recurrence.

In closing, the results of the present study confirmed that clinical disease stage and recurrence remain by far the most important predictors of prognosis and best guide to therapeutic decisions in OTSCC. Furthermore, the findings support that the histological risk model in this cohort was not an effective prognosis indicator, highlighting the heterogeneity of OTSCC and emphasizing the difficulty to standardize parameters that can be universally used as prognostic markers.

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CAPÍTULO 2

MYOFIBROBLASTS IN ORAL PREMALIGNANT LESIONS AND ORAL SQUAMOUS CELL CARCINOMAS

ABSTRACT

Several lines of evidence demonstrated that myofibroblasts in tumor stroma play an important role on development and progression of the malignant tumors, including oral squamous cell carcinoma (OSCC). The purposes of this study were evaluated myofibroblasts in oral dysplasias (mild, moderate and severe), OSCCs (well differentiated and poorly differentiated) and verrucous carcinoma and compare the presence of this cell type with fibrous hyperplasia with normal oral epithelium, and investigated the presence of myofibroblasts between OSCCs affecting young patients (<40 years) and older patients (>45 years). Myofibroblasts were detected by immunohistochemical expression of α smooth muscle actin (α-SMA) Both hyperplasias and dysplasias did not show myofibroblasts, whereas 62.2% cases of OSCCs demonstrated myofibroblasts in different densities. Poorly differentiated OSCCs demonstrated significantly higher density of myofibroblasts in comparison with well differentiated OSCCs or in comparison with verrucous carcinoma. Significant differences in the presence of myofibroblasts were not observed between OSCCs affecting young and old individuals. The results of present study demonstrated that immunodetection of myofibroblasts is not helpful on determining the malignant transformation potential of oral dysplasias, and is not associated with OSCCs affecting young patients.

Keywords: Oral squamous cell carcinoma, myofibroblast, dysplasia, verrucous carcinoma, young patients.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the eighth most prevalent malignant neoplasm worldwide, and mortality rates continue to be high in most countries, leading to an overall 5-year survival rate below 50% (MUSTAFA *et al*., 2005; XU *et al*., 2013). OSCC is considered a disease of middle-aged and older individuals, but an increasing incidence in young patients (age below 40 years) has been documented in recent years (TÚRI *et al*., 2013). The factors involved in the onset in young adults remain uncertain (RIBEIRO *et al*., 2009), and the biological behavior of OSCC affecting young patients has been a matter of controversy, since different authors have reported a more aggressive clinical course compared to those in adults over 45 years-old (SARKARIA et al., 1994; VARGAS et al., 2000; SIRIWARDENA et al., 2007; VERED et al., 2009), whereas others found no differences (ANNERTZ et al., 2002; SASAKI et al., 2005; PONTES et al., 2011; UDEABOR et al., 2012).

OSCCs are traditionally diagnosed at advanced stage, but invasive diseases are often preceded by the presence of clinically identifiable premalignant lesions, such as leucoplakias, eritroplakias and oral fibrous submucosa (LAPTHANASUPKUL et al., 2007; NAPIER & SPEIGHT, 2008 VILLA et al., 2011). Although presence and severity of epithelial dysplasia has been related to transformation into carcinoma, the mechanisms are poorly understood and transformation rates are extremely variable (HSUE, 2007). A growing body of evidences suggests that specific alterations in the underling connective tissue are necessary for tumor development and progression (NITTA et al., 2011; ALITALO & DETMAR, 2011). However, few reports have assessed the role of the subjacent connective tissue on malignant transformation of oral premalignant lesions. Rationality is based on fact that tumor and stromal cells exchange cytokines, extracellular matrix proteins and enzymes that promote growth directly through stimulation of proliferation and survival, as well as invasion via local proteolysis of the extracellular matrix (DE WEVER & MAREEL, 2003; DESMOULIÈRE et al., 2004; DE WEVER et al., 2008). Interestingly, we and others have demonstrated

that increased density of myofibroblasts, also called carcinoma-associated fibroblasts, in the stroma of OSCC correlated with lymph node metastasis and higher mortality (KELLERMANN et al., 2007; KELLERMANN et al., 2008; MARSH et al., 2011; DE-ASSIS et al., 2012).

In this study we investigated the presence of myofibroblasts in premalignant leucoplakias with different grades of dysplasia and in OSCC, and compared the presence of myofibroblasts in the stroma of OSCC of patients younger (below 40 years-old) and older (above 45 years).

MATERIAL AND METHODS

Tissue Samples

To address our specific objectives, the samples were divided in 2 groups. The first group consisted of 29 cases of fibrous hyperplasia with normal epithelium, 69 oral epithelial dysplasias with 24 classified as mild dysplasia, 26 as moderate dysplasia and 19 as severe dysplasia, 8 verrucous carcinoma and 37 OSCCs (19 well differentiated and 18 poorly differentiated). All samples were retrieved from the files of the Department of Oral Pathology, School of Dentistry, State University of Campinas, and new sections were cut from the paraffin blocks and stained with hematoxylin and eosin. Oral epithelial dysplasias and OSCCs were classified according to the World Health Organization (WHO) grading system.

The second group consisted of 29 cases of tongue OSCCs from patients under 40 years-old (27 well differentiated and 2 poorly differentiated) retrieved from four different Oral Pathology Centers at School of Dentistry, State University of Campinas, São Paulo State University (Araçatuba Dental School, Brazil), Clinic Center of Head and Neck (Guatemala) and The University of Sheffield (School of Clinical Dentistry, UK) in a 20-year period from 1988 to 2008. The mean age of young patients group at the time of the biopsy was 32.9 years with 51.7% of the patients reporting smoking and 62.1% drinking alcohol. The international approach was carried out to overcome the low incidence of OSCC in young patients and the focus on tongue intended to limit data variability. For

comparison proposes, a control group composed by tongue OSCCs from patients older than 45 years selected from the files of the Department of Oral Pathology, School of Dentistry, State University of Campinas. The group of older patients presented a mean age of 61.9 years, and most patients reported smoking (82.8%) and drinking alcohol (69.1%). Control group was matched in relation to tumor site, clinical stage of disease at diagnosis, and histopathological grade.

This study was approved by the Ethics Committee for Human Studies, Piracicaba Dental School and the South Sheffield Research Ethics Committee.

Immunohistochemistry

Immunohistochemistry was performed on 3 μm tissue sections using the avidin-biotin-peroxidase complex method. In essence, sections were deparaffinized and dehydrated using a graded series of ethanol. Sections were then subjected to antigen retrieval with 0.01 M citrate buffer pH 6.0 in an electric pressure cooker and incubation with 3% aqueous hydrogen peroxide for 15 min to quench endogenous peroxidase The sections were incubated with monoclonal mouse anti-α-SMA diluted 1:400 (Dako Corp., Carpenteria, CA, USA), followed by the LSAB detection system (Dako). Reactions were developed by incubating the sections with 0.6 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) containing 0.01% H_2O_2 and counterstained with Mayer's hematoxylin. The control reactions were performed by the exclusion of the primary antibodies. The presence of myofibroblasts was classified as negative, scanty or abundant, as described by Kellermann et al. 2007 in a blinded analysis performed by 3 of the authors.

Statistical analysis

For statistical purpose, negative and scanty samples were lumped together and compared with samples classified as abundant presence of myofibroblasts. Differences in the presence of myofibroblasts between groups were analyzed using the Fisher’s exact test at 5% significance (GraphPad Prism version 5.0).

RESULTS

The frequency of myofibroblasts in the different lesions is described in Table 1. Myofibroblasts were not found in fibrous hyperplasia with normal oral epithelium used as control and were also not detected in any of the 69 oral dysplasias (Fig. 1). The OSCCs cases showed positivity for myofibroblasts in 62.2% of the samples (Fig. 2). According to the histopathological differentiation, the presence of myofibroblasts was significantly higher in poorly differentiated OSCC in comparison with well differentiated ones (p=0,029). In well differentiated OSCCs, presence of myofibroblast was classified as negative in 9 (47.4%), scanty in 8 (42.1%) and abundant in 2 (10.5%), whereas in poorly differentiated OSCCs, 5 (27.8%) cases were classified as negative, 5 (27.8%) as scanty and 8 (44.4%) as abundant (Table 1). In the 8 samples of verrucous carcinoma, the presence of myofibroblasts was classified as negative in 7 (87.5%) and scanty in 1 (12.5%). The density of myofibroblasts was also significantly higher in poorly differentiated OSCCs than in verrucous carcinoma (p=0.031).

The OSCC affecting young patients showed positivity for myofibroblasts in 44.44% (n=16) of the samples. From those, 7 (19.44%) cases were classified as scanty and 9 (25%) cases as abundant (Table 2). In the lesions affecting older patients, positivity for myofibroblasts was seen in 66.66% (n=24) of the samples. The scanty presence of myofibroblasts was found in 13 (36.11%) cases and abundant presence of myofibroblasts was found in 11 (30.55%) cases (Table 2). By comparing the density of myofibroblasts in young and adult patients, no statistically significant difference could be obtained (p=0.59).

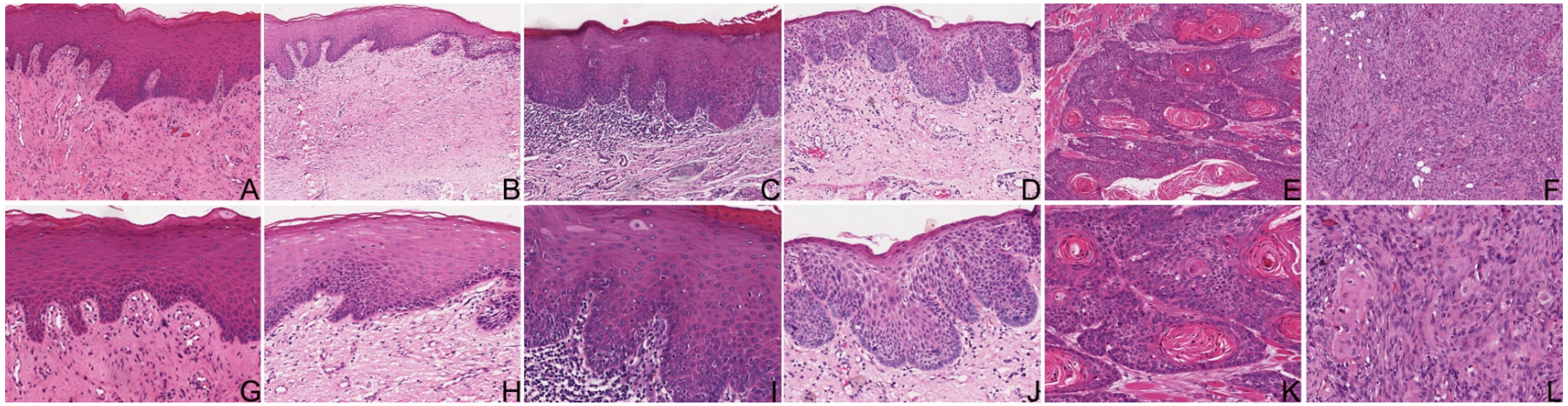

Figure 1 Representative images of the studied lesions. (A and G) fibrous hyperplasia, (B and H) mild dysplasia, (D and J) moderate dysplasia. (E and K) well differentiated OSCC, and (F and L) poorly differentiated OSCC.

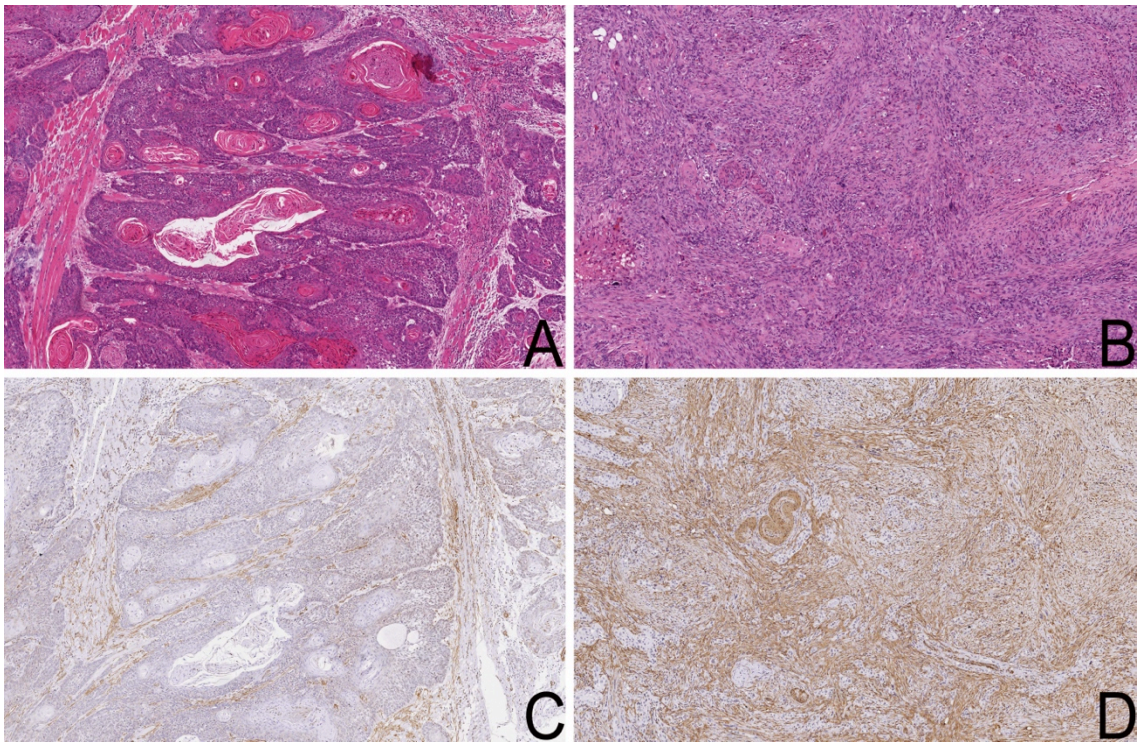

Figure 2. Histologic features and expression of myofibroblasts in representatives OSCC samples. (A and C) well differentiated OSCC showing a scanty expression of myofibroblasts and (B and D) poorly differentiated OSCC with abundant expression of myofibroblast.

Table 1. Presence of myofibroblast in all groups.

	Expression of myofibroblast		
	Negative n (%)	Scanty n (%)	Abundant n (%)
Hyperplasia	29 (100)	0	0
Mild dysplasia	24 (100)	0	0
Moderate dysplasia	26 (100)	0	0
Severe dysplasia	19 (100)	0	0
Well differentiated OSCC	9 (47.4)	8 (42.1)	2 (10.5)
Poorly differentiated OSCC	5 (27.8)	5 (27.8)	8(44.4)
Verrucous carcinoma	7 (87.5)	1 (12.5)	0

Table 2. Presence of myofibroblast in OSCC affecting young patients and older patients

	Myofibroblasts		
	Negative	Scanty	Abundant
	n (%)	n (%)	n (%)
Young patients			
Well Differentiated	10(71.42)	2(14.2)	2(14.2)
Moderately Differentiated	10(58.80)	3(17.64)	4(23.52)
Poorly Differentiated	0	2 (40)	3(60)
Total	20(55.55)	7(19.44)	9(25)
Older patients			
Well Differentiated	7(43.75)	4(25)	5(31.25)
Moderately Differentiated	5(33.33)	6(40)	4(26.66)
Poorly Differentiated	0	3 (60)	2 (40)
Total	12(33.33)	13(36.11)	11 (30.55)

DISCUSSION

Although several lines of evidence point toward an important role of the stroma in the development and progression of the malignant tumors, the specific mechanisms associated with its activation and effects on regulation of the tumorigenesis are not fully understood (TUXHORN et al., 2002). One of the most evident phenomenons in tumor stroma is the acquisition of myofibroblasts. The transdifferentiation of myofibroblast has been considered a crucial and early event in tumorigenesis, and is mediated by growth factors and cytokines released by tumor cells (BAGLOLE et al., 2006; MARSH et al., 2011; THODE et al., 2011) Myofibroblasts were originally identified in granulation tissues as modified fibroblasts with prominent rough endoplasmatic reticulum and Golgi apparatus producing collagen, abundant myofilaments characterized by the presence of α-SMA, and fibronexus junctions (SINGER, 1984). Later, it was showed that myofibroblasts may control several physiological and pathological events via secretion of an extensive repertoire of cytokines, growth factors, chemokines, hormones, neurotransmitters, inflammatory mediators, adhesion proteins, and most abundantly extracellular matrix proteins (POWELL, 2000, DESMOULIÈRE et al., 2004, POWELL et al., 2005). Several studies reported myofibroblasts in invasive breast, throat, larynx and oral cavity cancers (BARTH et al., 2004; YAZHOU et al., 2004; CIMPEAN et al., 2005; KELLERMANN et al., 2007; VERED et al., 2009; CHAUDRARY et al., 2012) and few studies have evaluated myofibroblasts in oral dysplasia (KELLERMANN et al., 2007; VERED et al., 2009; CHAUDRARY et al., 2012), oral verrucous carcinoma (CHAUDRARY et al., 2012) and OSCC (BARTH et al., 2004; LEWIS et al., 2004; KELLERMANN et al., 2007; VERED et al., 2007; KELLERMANN et al., 2008; VERED et al., 2009; SOBRAL et al., 2011; CHAUDHARY et al., 2012). Studies investigating the stromal constituents of OSCC affecting young individuals and their relevance for biological behavior of the tumors are very scarce (SASAKI et al., 2005; FALAKI et al., 2011), and specifically studying the presence of myofibroblasts, to date, has never been conducted.

In the present study myofibroblasts were not found in hyperplasias with normal epithelium and in any of the 69 oral dysplasias. Previous studies did not find myofibroblasts in oral normal mucosa and oral dysplasias, highlighting the necessity of the molecular cross talk between stromal elements and tumor cells during invasion of the connective tissue for the emergency of myofibroblasts (BARTH et al., 2004; ETEMAD-MOGHADAM et al., 2009; KELLERMANN et al., 2007; DE-ASSIS et al., 2012; OTRANTO et al., 2012). However, few studies demonstrated myofibroblasts in severe oral dysplasias (SEIFI et al., 2010; CHAUDHARY et al., 2012) and in other anatomic sites, including breast, bladder, cervix and intestinal mucosa (SAPPINO et al., 1988; CINTORINO et al., 1991; POWELL et al., 2005; CHAUHAN et al., 2003; KURODA et al., 2006; SHIMASAKI et al., 2006). Seifi et al., (2010) demonstrated that the expression of myofibroblast in the oral dysplasias is lower than in the OSCCs, showing an increase in the number of myofibroblasts during the carcinogenesis. Similarly, Chaudhary et al. (2012) showed that the presence of myofibroblasts increases as the disease progresses from oral high risk epithelial dysplasias to verrucous carcinoma and to invasive OSCC.

The present study also demonstrated that myofibroblasts were found with frequency in the stroma of invasive OSCCs. This reinforces the hypothesis that interactions between tumor cells and tumor microenvironment is important for oral carcinogenesis (WEAVER & GILBERT, 2004; BEACHAM & CUKIERMAN, 2005). Transformed myofibroblasts in OSCCs may play an active role in disease progression by both autocrine effect on tumor stroma and paracrine effect on malignant epithelial cells through tumor-stromal interactions (KELLERMANN et al., 2008; CHAUDHARY et al., 2012). The neoplastic changes that occur in the epithelium are followed by changes in the stroma that are caused by factors such as PDGF and TGF-β1 from stromal surrounding tumor cells, which in turn promote the differentiation of fibroblasts into myofibroblasts (VARAYOUD et al., 2001). This happens as a result of induction by a variety of cytokines from cancerous cells

(DESMOULIÈRE et al., 2004). OSCC cells have been shown to secrete high amounts of cytokines and growth factors such as TGF-β1, TGF-α, IGF-related factors, and interleukins (CHAUDHARY et al., 2012). TGF-β1, in particular, is responsible for the fibroblast to myofibroblast transdifferentiation in experimental models, how we showed in our previous study (KELLERMANN et al., 2008).

The presence of myofibroblasts was significantly higher in poorly differentiated OSCC in comparison with well differentiated ones. These findings may suggest that the loss of tumoral differentiation affects the number of myofibroblasts in the tumoral stroma (KAWASHIRI et al., 2009; CHAUDHARY et al., 2012) and that higher the number of myofibroblasts, more invasive is the tumor phenotype (CHAUDHARY et al., 2012). The density of myofibroblasts was also significantly higher in poorly differentiated OSCCs than in verrucous carcinoma and this result was consistent with the fact of the verrucous carcinoma be a special form of well-differentiated squamous cell carcinoma with specific clinical and histological features, as slow growth, local invasion and unlikely to spread metastasize (ALKAN et al., 2010; CHAUDHARY et al., 2012). Since the well differentiated OSCC closely resembles normal squamous mucosa and the prognosis is better than less-well-differentiated neoplasms (KANG et al., 2011). However, our findings are not in agreement with previous studies (Kellerman et al., 2007; Etemad Moghadam et al., 2009), which did not found correlation between presence of myofibroblasts and tumor differentiation. The grading by differentiation of the tumor is really of limited prognostic value, as compared with the pattern of invasion (BARNES et al., 2005). However, studies with a large sample are required to confirm the association among the increase of myofibroblasts and the loss of differentiation of the OSCCs.

The results obtained in the evaluation of the presence of myofibroblast in OSCC affecting young patients revealed that there was no significant difference between the presence of myofibroblasts in young patients if compared to older individuals. Several uncertainties are related about the biological and clinical

behavior of the OSCC affecting young patients (TÚRI et al., 2013). These cases have initially been considered to represent a more aggressive disease than those diagnosed in older patients, also not showing a strong association with tobacco use and alcohol intake, suggesting that alternative etiologic factors would be involved with its onset (SASAKI et al., 2005; RIBEIRO et al., 2007; HIROTA et al., 2008). However, recent investigations proved that no significant difference can be found in the prognosis, survival and treatment response of young patients afflicted by OSCC when compared to older ones, what is further supported by most of the molecular researches evaluating the epithelial component of OSCC that failed to obtain significant differences between both groups of patients, also suggesting that no relevant difference would exist (SIRIWARDENA et al., 2007; WARNAKULASURIYA et al., 2007; THOMAS et al., 2012; BENEVENUTO et al., 2012). Considering that myofibroblasts could induce a highly vasculogenic stroma (KAWASHIRI et al., 2009), the results here described with no significant difference in the myofibroblastic component between OSCC of young and old patients, reinforce the results obtained by Benevenuto et al. (2012), where no significant difference could be seen in the angiogenic index of OSCC of both groups. Hence, taking into account this study, it can be speculated that if a different behavior would in fact exist for cases affecting young patients, such characteristic would be more prone to be related to molecular features of the neoplastic epithelial compartment, rather than the myofibroblast in the stromal compartment.

In conclusion, our results showed that myofibroblast expression is not associated with oral dysplasias, however poorly differentiated OSCCS showed a higher density of myofibroblasts than well differentiated OSCCs. In addition, our findings revealed that no significant difference exist between the myofibroblast presence in the stromal components of OSCC affecting young and old individuals. However, studies should be carried out with a larger sample to confirm the association of increased presence of myofibroblast with loss of histological differentiation and for better understanding the biological features of OSCC

affecting young patients.

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Conclusão

1. Características clínicas como o tamanho do tumor (estadio T), a presença de metástase regional (estadio N) e a recorrência são melhores fatores preditivos de prognóstico de CECs orais que o risco histológico;

2. A presença de miofibroblastos não foi detectada em amostras de hiperplasia com epitélio oral normal e nem em amostras de displasias orais (leve, moderada e severa), mas foi detectada com frequência em CECs orais;

3. Tumores pobremente diferenciados apresentam uma densidade significativamente maior de miofibroblastos do que os tumores bem diferenciados;

4. Não há diferença na densidade de miofibroblastos entre CECs orais que afetam jovens e idosos, sugerindo que a presença de miofibroblastos no componente estromal não é responsável pelas diferenças entre os dois grupos.

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