UNESP - Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara

Heitor Albergoni da Silveira

Caracterização imunoistoquímica comparativa de subgrupos de células dendríticas e oncogênese viral no carcinoma espinocelular oral e orofaríngeo

Araraquara

2020

UNESP - Universidade Estadual Paulista

Faculdade de Odontologia de Araraquara

Heitor Albergoni da Silveira

Caracterização imunoistoquímica comparativa de subgrupos de células dendríticas e oncogênese viral no carcinoma espinocelular oral e orofaríngeo

Dissertação apresentada ao programa de Pós-Graduação em Ciências Odontológicas, Área de concentração: Diagnóstico e Cirurgia, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista para o título de Mestre em Ciências Odontológicas.
Orientador: Jorge Esquiche León

Araraquara

2020

Silveira, Heitor Albergoni da
Caracterização imunoistoquímica comparativa de subgrupos de células dendríticas e oncogênese viral no carcinoma espinocelular oral e orofaríngeo / Heitor Albergoni da Silveira. -- Araraquara: [s.n.], 2020
88 f.; 30 cm.

Dissertação (Mestrado em Ciências Odontológicas) – Universidade Estadual Paulista, Faculdade de Odontologia
Orientador: Prof. Dr. Jorge Esquiche León

1. Carcinoma de células escamosas 2. Boca 3. Orofaringe 4. Papillomaviridae 5. Imuno-histoquímica 6. Células dendríticas I. Título

Ficha catalográfica elaborada pela Bibliotecária Marley C. Chiusoli Montagnoli, CRB-8/5646
Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara
Diretoria Técnica de Biblioteca e Documentação

Heitor Albergoni da Silveira

Caracterização imunoistoquímica comparativa de subgrupos de células dendríticas e oncogênese viral no carcinoma espinocelular oral e orofaríngeo

Comissão julgadora

Dissertação para obtenção do grau de Mestre em Ciência Odontológicas

Presidente e orientador: Prof. Dr. Jorge Esquiche León

2º Examinador: Prof. Dr. Carlos Rossa Junior

3º Examinador: Profa. Dra. Ana Terezinha Marques Mesquita

Araraquara, 19 de fevereiro de 2020

DADOS CURRICULARES

Heitor Albergoni da Silveira

NASCIMENTO: 20/06/1995 – Bernardino de Campos, São Paulo, Brasil
FILIAÇÃO: Ademilson Andrei da Silveira e Isabel Cristina Albergoni

2013-2016: Graduação em Odontologia, Universidade do Sagrado Coração, Bauru, São Paulo.

2017-2018: Aprimoramento em Estomatologia, Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista, Araraquara, São Paulo**.**

2018-2020: Mestrado em Ciências Odontológicas – área de concentração: Diagnóstico e Cirurgia, Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista, Araraquara, São Paulo**.**

UNESP - Universidade Estadual Paulista

Faculdade de Odontologia de Araraquara

Heitor Albergoni da Silveira

Caracterização imunoistoquímica comparativa de subgrupos de células dendríticas e oncogênese viral no carcinoma espinocelular oral e orofaríngeo

Orientador: Prof (a) Dr (a) Jorge Esquiche León

Assinatura Orientador (a):

Assinatura Aluno (a):

Araraquara, 19 de Fevereiro de 2020 .

Dedico esse trabalho à minha vózinha Josefa (in memorian)

Te amarei todos os dias da minha vida

AGRADECIMENTOS

À **Deus** em primeiro lugar, por sempre me iluminar e me dar proteção nos momentos mais difíceis da minha vida, sem Ele esse sonho não seria possível.

Aos meus avós **Osmar** e **Elza,** obrigado por tanto amor e carinho. Vocês são meus maiores exemplos.

Aos meus pais **Ademilson** e **Isabel Cristina** por sempre me apoiarem em todas minhas decisões, por não medirem esforços para me ajudar. Agradeço imensamente por todo amor e suporte. Gratidão e amor eterno por vocês.

À minha irmã **Amanda**, por toda amizade e cumplicidade, me acompanhou em todos os momentos bons e nem tão bons da vida e sempre esteve do meu lado me apoiando em todas as minhas decisões, e obrigado também por nos dar o presente mais lindo que é a **Isabela**. Amo muito vocês duas!

Ao meu orientador Prof. Dr. **Jorge Esquiche León**, obrigado por toda a paciência e amizade que construímos no decorrer desses anos, por toda a disponibilidade e vontade em me ensinar e me forçar a ser cada dia melhor, agradeço imensamente a confiança depositada em mim para o desenvolvimento desse projeto. Minha eterna admiração e gratidão.

À Faculdade de Odontologia de Araraquara – Universidade Estadual Paulista, representada pelo digníssimo Diretor Prof. Dr. **Edson Alves de Campos**.

À Profa. Dra. **Andreia Bufalino**, por ser um exemplo de profissional e por ser tão solicita a todos nós alunos da área do Diagnóstico bucal, sem a sua ajuda tudo seria mais difícil.

Aos meus grandes amigos **Darcy Fernandes**, **Vinicius Krieger Costa Nogueira** e **Igor Paulino Soares**, obrigado por toda irmandade nessa jornada, por todas as risadas e todo carinho. Levarei vocês sempre comigo.

À minha dupla de Pós-Graduação, **Camila de Oliveira Barbeiro** pela amizade e parceria por dividir todos esses momentos do mestrado comigo.

A todas as professoras da disciplina de Estomatologia, **Elaine Maria Sgavioli Massucato**, **Mirian Onofre**, **Claudia Maria Navarro** e **Luciana Yamamoto de Almeida**.

Aos meus amigos da Pós-Graduação, **Evânio Vilela da Silva**, **Tulio Morandin Ferrisse**, **Maria Leticia Lança**, **Larissa Natiele Miotto** e **Audrey Foster Lefort Rocha**.

A FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (Processo nº 2018/12734-2) pela concessão da bolsa de mestrado e auxílio financeiro (Processo nº 2016/11419-0).

Silveira HA. Caracterização imunoistoquímica comparativa de subgrupos de células dendríticas e oncogênese viral no carcinoma espinocelular oral e orofaríngeo [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2020.

RESUMO

O carcinoma espinocelular (CEC) de cabeça e pescoço (CECCP) é o quinto tipo de câncer mais comum e a sexta causa de morte por câncer. Recentes estudos enfatizam o CEC oral (CECO) como uma entidade distinta do CEC de orofaringe (CECorof), com este último apresentando melhor prognóstico e estreitamente associado com infecção pelo papilomavírus humano (HPV). A etiologia do CECCP é multifatorial; porém, o CECO está relacionado com abuso do tabaco e álcool, enquanto o CECorof está frequentemente associado com infecção pelo HPV. As células dendríticas (CDs) são importantes células as quais regulam repostas imunes, incluindo aquelas vinculadas à tumorigênese, estabelecendo conexão entre o sistema imune inato e adaptativo. Estão divididas em dois grupos: CDs mieloides (CDmi) e CDs plasmocitóides (CDp), incluindo CDs imaturas (CDim) e CDs maduras (CDm). O objetivo do nosso estudo foi analisar comparativamente, através da técnica de imunoistoquímica (IQ) e hibridização in situ (HIS), a infiltração de CDmi e CDp, incluindo subgrupos de CDim e CDm, no CECO (n=109) e CECorof (n=126), bem como analisar a oncogênese viral (HPV amplo espectro, alto risco (ARHPV) e baixo risco (BRHPV) oncogênico e vírus Epstein-Barr [VEB]) por HIS. O CECOrof (25%) comparado com o CECO (11%) mostrou associação significativa com o HPV. No CECorof e no CECO, 19 e 7 mostram positividade para ARHPV (somente), 6 e 3 BRHPV (somente) e 3 e 2 ARHPV/BRHPV (co-infecção), respectivamente. Os tumores HPV-positivos comparados com os HPV-negativos apresentaram índice proliferativo significativamente maior através dos marcadores Ciclina D1 e Ki-67. O CECO associado ao ARHPV e ao BRHPV apresentou maior taxa de sobrevida global. No geral, os marcadores para imDC, foram significativamente mais frequentes no CECorof do que no CECO. Diferentemente, CECO e CECorof apresentaram maior número de marcadores de CDs submucosas (CDsub). Ambas as neoplasias apresentaram quantidades semelhantes de CDp ativadas. O grupo HPV+ mostrou um número maior de CDs em ambas as neoplasias do que o grupo HPV-. Vale ressaltar que um número significativamente maior de células CD207+ e CD123+ foi observado no CECorof HPV+ do que no CECO HPV+. Concluimos que diferente do CECO, nossos resultados mostram no CECorof predominância de CDim, com perfil de ativação de células imunes. A presença do HPV parece mostrar associação com a infiltração de CDs em ambas as neoplasias, sugerindo respostas imunes antivirais no CECorof HPV+.

Palavras – chave: Carcinoma de células escamosas. Boca. Orofaringe. Papillomaviridae. Imuno-histoquímica. Células dendríticas.

Silveira HA. Comparative immunohistochemistry characterization of subgroups of dendritic cells and viral oncogenesis in oral and oropharyngeal squamous cell carcinomas [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2020.

ABSTRACT

Head and neck squamous cell carcinoma (SCC) (HNSCC) is the fifth most common type of cancer and the sixth leading cause of cancer death. Recent studies emphasize oral SCC (OSCC) as an entity distinct from oropharyngeal SCC (OPSCC), with the latter having a better prognosis and closely associated with human papillomavirus (HPV) infection. The etiology of HNSCC is multifactorial; however, OSCC is related to tobacco and alcohol abuse, while OPSCC is often associated with HPV infection. Dendritic cells (DCs) are important cells that regulate immune responses, including those linked to tumorigenesis, establishing a connection between the innate and adaptive immune systems. They are divided into two groups: myeloid DCs (myCD) and plasmacytoid DCs (pDC), including immature DCs (imDC) and mature CDs (mDC). The aim of our study was to comparatively analyze, through the immunohistochemistry (HIS) and in situ hybridization (ISH) technique, the infiltration of myCD and pDC, including subgroups of imDC and mDC, in the OSCC (n= 109) and OPSCC (n= 126), as well as analyzing viral oncogenesis (wide-spectrum HPV, high-risk (HRHPV) and low-risk (LRHPV) oncogenic and Epstein-Barr virus [EBV]) by ISH OPSCC (25%) compared to OSCC (11%) showed a significant association with HPV. In OPSCC and OSCC, 19 and 7 shows positivity for HRHPV (only), 6 and 3 LRHPV (only) and 3 and 2 HRHPV/LRHPV (co-infection), respectively. HPV-positive tumors compared to HPV-negative tumors showed a significantly higher proliferative index through the cyclin D1 and Ki-67 markers. The OSCC associated with HRHPV and LRHPV had the highest overall survival rate. Overall, markers for imDC were significantly more frequent in OPSCC than in OSCC. In contrast, OSCC and OPSCC had a higher number of subDC markers. Both neoplasms showed similar amounts of activated pCD. The HPV group showed a greater number of CDs in both neoplasms than the HPV-. It is noteworthy that a significantly higher number of CD207+ and CD123+ cells was observed in HPV-associated OPSCC than in HPV-associated OSCC. We concluded that unlike OSCC, our results show a predominance of imDCs, with an activation profile of immune cells, in OPSCC. The HPV status seems to show an association with the infiltration of DCs in both neoplasms, suggesting antiviral immune responses in the OPSCC associated with HPV.

Keywords: Squamous cell carcinoma. Mouth. Oropharynx. Papillomaviridae. Immunohistochemistry. Dendritic cells.

SUMÁRIO

1 INTRODUÇÃO

1.1 Carcinoma Espinocelular da Região de Cabeça e Pescoço: Epidemiologia

O carcinoma espinocelular (CEC) da região de cabeça e pescoço (CECCP) é um grupo heterogêneo de neoplasias malignas provenientes da superfície mucosa da cavidade oral, nasofaringe, orofaringe, hipofaringe, laringe, seios paranasais e pele[1]. Atualmente, a Organização Mundial da Saúde (OMS, 2017)[2], tem classificado o CEC oral (CECO) e CEC de orofaringe (CECorof) como duas neoplasias distintas, principalmente devido as suas particularidades anatômicas de cada região, na prevalência de infecção pelo HPV e especialmente por diferenças no prognóstico entre estes dois tumores[2].

Para o Brasil, segundo o Instituto Nacional do Câncer (INCA)[3], estimam-se 11,200 casos novos de câncer da cavidade oral em homens e 3,500 em mulheres para cada ano do biênio 2018-2019. Esses valores correspondem a um risco estimado de 10,86 casos novos a cada 100 mil homens, ocupando a quinta posição; e de 3,28 para cada 100 mil mulheres, sendo o 12º mais frequente entre todos os cânceres[3]. Além disso, estimam-se 6,390 casos novos de câncer de laringe em homens e 1,280 em mulheres para cada ano do biênio 2018- 2019. O risco estimado será de 6,17 casos a cada 100 mil homens, ocupando a oitava posição; e a 16ª mais frequente com 1,20 casos a cada 100 mil mulheres[3].

O CECO é considerado a sexta neoplasia epitelial maligna mais comum[4]. São tumores com comportamento clínico agressivo, apresentando altas taxas de morbimortalidade, apesar dos significativos avanços nos protocolos terapêuticos alcançados nas últimas décadas[5]. Os homens são afetados em maior proporção quando comparado com as mulheres, podendo ser precedidas por desordens potencialmente malignas (DPMs), dentre elas estão a leucoplasia oral (LO), leucoplasia verrucosa proliferativa (LVP), eritroplasia, incluindo ainda a fibrose submucosa, anemia fanconi e disqueratose congênita[6]. O CECO compreende aproximadamente 90-95% das lesões malignas que atingem a cavidade oral[7]. Aproximadamente, entre 10-30% dos casos de CECO apresentam infecção pelo HPV[1], com uma taxa de sobrevida geral em 5 anos de 50%[8]. Diferentemente, mais de 70% dos casos de CECorof estão associados com infecção pelo HPV, a

maioria deles (87%) positivos para HPV de alto risco (HPV16/18), sendo a taxa de sobrevida geral em 5 anos de 75-80%[9-12].

1.2 Fatores de Risco para o CECO e CECorof

A etiologia do CECCP é considerada multifatorial, mas está intimamente relacionada com o abuso do tabaco e álcool e a infecção pelo HPV, especialmente o tipo 16/18, que representam os subtipos de HPV de alto risco oncogênico[13,14]. O consumo do tabaco e álcool parece ser dose/dependente e tempo/dependente e a combinação desses dois fatores leva ao efeito sinérgico, aumentando as chances de desenvolvimento do CEC[7,15,16]. O tabaco foi considerado pela *International Angency for Research on Cancer (IARC)*[17] como um agente cancerígeno do grupo 1, que indica alto risco[17]. Outros fatores de risco vêm sendo investigados, tais como a desnutrição geral[18], o baixo nível socioeconômico e uma higiene oral precária[19,20]. Notavelmente, o fator de risco mais frequentemente associado com o CECorof HPV+ foi o comportamento sexual e para o CECorof HPV- foi o consumo de álcool e tabaco[2].

Existem mais de 200 tipos de HPV identificados, os quais são classificados em grupo de alto ou baixo risco, de acordo com o seu potencial tumorigênico[21,22]. O HPV é um pequeno vírus de DNA com tropismo específico para o epitélio escamoso. Em uma infecção persistente, a proteína E2 viral controla rigorosamente a expressão das principais oncoproteínas virais E6 e E7. Estas proteínas são os principais impulsionadores da tumorigênese por inativação de duas importantes moléculas supressoras de tumores, proteína retinoblastoma (pRb) e p53. A inibição das proteínas supressoras de tumores p53 e pRb altera as vias do ciclo celular que regulam a proliferação celular, a apoptose, bem como a estabilidade genética, o que pode levar à formação de lesões epiteliais[1].

Interessantemente, o CECorof afeta preferencialmente uma população mais jovem, não fumante e que não consomem álcool [13]. Além disto, o CEC orof HPV+ mostra uma resposta mais eficiente aos tratamentos anti-neoplásicos, principalmente a radioterapia, quando comparados com CEC orof HPV-[7,23,24]. Com base em estudos envolvendo infecção cervical pelo HPV, a maioria dos indivíduos infectados terá um curso assintomático, com a liberação do vírus ocorrendo em 90% deles dentro de 1 ou 2 anos, já os outros 10% terão infecção persistente e um

risco aumentado em desenvolver câncer. Destes 10%, cerca de metade destes casos desenvolverá neoplasia maligna[25].

Assim, diante do exposto, é evidente que vários estudos mostram que o CECO e CECorof apresentam etiopatogenia, características clínicas, fatores de risco, protocolos terapêuticos e prognóstico diferentes[7,26].

1.3 Características Clinicopatológicas

1.3.1 Características clinicopatológicas do CECO

O CECO pode apresentar diversas formas clínicas variando de uma placa branca até lesões ulcerativas, exofíticas, com bordas elevadas e base endurecida. Nas fases iniciais é assintomática e, posteriormente com o avanço da doença, podem-se observar sinais e sintomas de desconforto, como dor e mobilidade reduzida da língua. A região de maior acometimento na cavidade oral é a língua, soalho e mucosa jugal[2,27,28].

O exame histopatológico do CECO mostra proliferação atípica das células epiteliais, seguindo um percurso de invasão do tecido conjuntivo. Pérolas de queratina, pleomorfismo celular, figuras de mitoses atípicas são frequentemente observadas, aumentando com o grau histológico do tumor[29]. A classificação do grau de diferenciação do CECO segue os critérios propostos pela Organização Mundial da Saúde (OMS), podendo ser bem diferenciados (grau I), moderadamente diferenciados (grau II) e pobremente diferenciados (grau III). Algumas vezes são necessários estudos complementares como a IQ, HIS e/ou genética molecular, para estabelecer o grau de diferenciação e tipo histopatológico[2].

1.3.2 Característica clinicopatológicas do CECorof

Os tumores que atingem a região de orofaringe são frequentemente encontrados na base da língua, região tonsilar e palato mole, clinicamente se apresentando como massas ulceradas ou extensas ulcerações delimitadas por mucosa eritematosa e irregular. Os principais sintomas relatados por pacientes com CECorof incluem dor de garganta e disfagia[7].

Histologicamente, a orofaringe apresenta epitélio escamoso, tecido linfóide e glândulas salivares menores, o que acaba possibilitando o desenvolvimento de diferentes tipos histológicos de neoplasias. Nessa região, aproximadamente 90-95% dos tumores malignos são CEC. Interessantemente, os CECorof HPV+ surgem do epitélio das criptas ou reticular o qual reveste as criptas tonsilares. O tumor prolifera envolvendo o revestimento epitelial de superfície, sendo a displasia epitelial raramente identificada. Os ninhos tumorais infiltrativos são frequentemente incorporados no estroma linfático e podem ser permeados por células linfoides. As células tumorais apresentam uma alta taxa mitótica e/ou apoptótica, sendo que a sua morfologia lhe confere uma aparência basalóide. A classificação histopatológica do CECorof inclui CEC convencionais tais como ceratinizante, não ceratinizante e híbrido, e variantes tais como basaloide, papilar e linfoepitelial, entre outros[30,31]. Interessantemente, existem trabalhos considerando que o CECorof HPV+, pelo comportamento clínico e prognóstico, pode ser melhor considerado como um CEC bem diferenciado, apesar da aparência imatura e da falta de produção de queratina[30,31].

Em relação à infecção por HPV em CECorof deve se levar em consideração a região geográfica em que o estudo é realizado, trabalhos com população norte americana mostraram resultados de positividade do HPV nessas neoplasias em uma porcentagem de 54,7%, enquanto na américa central e do Sul esses valores representaram 14,9%, evidenciando um perfil de baixa infecção pelo HPV. Os continentes da Ásia, Oceania e Europa demostraram taxas de positividade para o vírus de 45%, 42.1% e 36.2%, respectivamente. Segundo uma revisão sistemática realizada por Ndiaye et. al.[32] (2014). Trabalhos realizados com a população brasileira mostra que a associação entre o CECOrof e o HPV representam de 5,6% a 25,6%, sendo semelhante aos achados de países como Cuba (15.4%), Tailândia (14.5%) e Polônia (10.7%)[33-38].

O vírus Epstein-Barr (VEB) também demonstra um potencial carcinogênico, estando associado com CEC nasofaringe e linfoma não-Hodgkin[39]. Szkaradkiewicz et al.[39] (2002) realizaram um estudo para identificação de VEB-DNA em amostras de CECorof, e revelou uma taxa de 86% mostraram positividade para o VEB, sugerindo que as persistências desse vírus nas células orofaríngeas, sob

condições genéticas e ambientais, podem promover a carcinogênese[39]. No entanto, neste estudo, não foi realizado HIS (em tecidos) para a detecção do VEB.

No presente estudo, diferentemente do estudo de Szkaradkiewicz et al.[39] (2002), avaliamos por HIS (EBER) todos os CECCP incluídos na amostra, focando especialmente resultados nos CECs indiferenciados HPV-.

1.4 Prognóstico

Atualmente os tumores primários de cavidade oral e orofaringe são classificados segundo o sistema de estadiamento clínico, proposto pelo Comitê Americano de Câncer (*American Joint Committee on Cancer – AJCC*), conhecido com TNM, o qual leva em consideração o tamanho do tumor primário (T), linfonodos envolvidos (N) e metástase a distância (M)[7]. Tal classificação tem sido utilizada como ferramentas no estabelecimento do tratamento e prognóstico destes tumores. Contudo, o CECorof HPV+ parece apresentar um prognóstico mais favorável quando comparado com o CECorof HPV- e CECO associado ao uso de tabaco e/ou álcool, mesmo quando existe o comprometimento de linfonodos regionais[7,40,41]. Assim, tem sido sugerido que se faça parte do estadiamento clínico a história do uso de tabaco e/ou álcool e a presença de HPV[7,40].

De forma geral, o CECO é agressivo e apresenta propensão à invasão local e metástase para linfonodos; sendo que a presença de características histopatológicas como padrão de invasão não coesivo, invasão perineural e linfovascular, revelam um pior prognóstico. Além disso, os indivíduos fumantes e etilistas apresentam um risco 20 vezes maior para o desenvolvimento de recidivas ou segundos tumores primários na cavidade bucal ou no trato aerodigestivo quando comparados aos indivíduos não fumantes e não etilistas, especialmente, quando mantêm o consumo de tabaco e álcool após o diagnóstico do tumor primário[42,43].

1.5 Tratamento

O tratamento do CECO e CECorof deve ser planejado a partir do seu estadiamento clínico. As lesões primárias de orofaringe têm como tratamento indicado a cirurgia e a radioterapia isoladas, e as lesões mais avançadas exigem

combinações das terapias anti- neoplásicas como cirurgia seguida de radioterapia ou radioterapia inicial e quimioterapia concomitante[7]. Estudos mostram que a radioterapia isolada em CECorof HPV+ tem melhor resultado quando comparado com o CECorof HPV-[9,44,45,46,47]. Resultados semelhantes foram encontrados em um estudo *in vivo,* o qual mostrou que os CECs HPV+ foram mais sensíveis à radioterapia e quimioterapia em camundongos imunocompetentes, quando comparados com CECs HPV-[23,48]. Outros estudos mostraram também que o CECorof HPV+ tem uma melhor resposta à radioterapia, e sugerem que essa característica pode estar associada à imunovigilância vírus-específica e à ausência de campos cancerizáveis e não exclusivamente ao fato dessas lesões serem mais radiossensíveis[9,44].

O tratamento do CECO é planejado de acordo com o seu estadiamento clínico. Geralmente, esses tumores se apresentam em estágios avançados devido à demora do paciente na busca do tratamento. A cirurgia isolada ou radioterapia é indicada para as lesões iniciais. Quando a neoplasia está em estágio mais avançado o tratamento é a cirurgia radical associada à radioterapia, podendo ser empregada também a quimioterapia em pacientes com envolvimento sistêmico após a realização do procedimento cirúrgico[49].

1.6 Sistema Imune, Células Dendríticas e Oncogênese

1.6.1 Sistema imune

Nos últimos anos estudos mostraram que o sistema imune desempenha um papel fundamental no controle e progressão do tumor. Células imunes infiltrantes nos tumores, incluindo linfócitos T e B, macrófagos e neutrófilos podem regular respostas imunes, inibindo ou estimulando o crescimento tumoral[24,50]. De fato, a caracterização da resposta imune adaptativa mostrou ser uma ferramenta prognóstica importante em uma ampla gama de carcinomas, potencialmente ainda mais relevante devido ao desenvolvimento de protocolos imunoterapêuticos[51,52].

Os linfócitos T regulatórios (LTregs) são responsáveis não somente pelo controle de linfócitos autoreativos, mas também pela redução da resposta imune ao antígeno tumoral. Os LTregs (CD4+/CD25+/Foxp3+) representam 2-4% do total de LTCD4+, sendo fundamentais na manutenção da autotolerância periférica.

Embora o mecanismo de ação não seja completamente compreendido, LTregs regulam as funções de LTCD4+, LTCD8+, células natural killer (NK), CDs e macrófagos durante a resposta imune contra patógenos, autoantígenos e tumores[24]. Similar com LTregs, porém diferentemente dos macrófagos M1, os macrófagos M2 também têm mostrado um perfil anti- inflamatório e pró-tumoral[53]. Vários estudos avaliando o envolvimento dos LTregs e macrófagos M2 na progressão tumoral, mostram um aumento no número de destas células no sangue periférico de pacientes com câncer de pulmão, próstata, mama e cabeça e pescoço[24,53,54], usualmente associado com prognóstico ruim[53]. Outros estudos têm demonstrado que a depleção de LTregs e macrófagos M2 tem potencial terapêutico nos pacientes com câncer[53,55,56]. Em pacientes com tumores de cabeça e pescoço, a resposta imune antitumoral é prejudicada e a progressão está associada à disfunção imune grave[57].

1.6.2 Células dendríticas

As CDs são importantes na iniciação e regulação das respostas imunes. Estão presentes em quase todos os tecidos periféricos, incluindo pele e mucosa[58-61]. As CDs não somente ativam linfócitos T e B, mas também células NK e produzem interferons (IFNs), estabelecendo uma conexão entre o sistema imune inato e adaptativo (Han et al., 2017). A ativação (ou maturação) de CDs resulta em imunidade, uma vez que, dependendo da natureza do estímulo de ativação, as CDs podem induzir respostas imunes mediadas por LTh1 e células de langerhans (CLs)[62]. Em contraste, CDs inativas ou CDs recebendo estímulos inibitórios, como IL-10 e/ou corticosteróides, induzem tolerância imunológica via depleção de linfócitos T e proliferação de LTregs. Assim, a resposta imunológica é dependente do estado de ativação da CDs: as CDs maturas (CDm) protegem o organismo de neoplasias ou patógenos, enquanto as CDs imaturas (CDim) induzem tolerância imunológica[63]. O processo de vigilância imunológica do câncer é um importante processo de proteção do hospedeiro para inibir a carcinogênese e para manter a homeostase celular[64]. Neste contexto, as CDs são conhecidas por desempenhar um papel central na regulação de respostas imunológicas inatas e adaptativas, incluindo imunidade antitumoral[65].

1.6.3 Células dendríticas da mucosa oral (CDMO)

Vários estudos sugerem que as CDMO possuem propriedades tolerogênicas, tais como a produção de citocinas regulatórias (IL-10 e TGF-β), geração de LTregs e alteração da resposta Th1/Th2[66]. Imunidade atenuada nesse contexto faz sentido, porque as superfícies mucosas estão expostas continuamente a diversas substâncias inócuas[67]. Mascarell et al.[68] (2008) realizaram análises mais detalhadas das CDMO e identificaram quatro subconjuntos de CDMO: (i) CLs CD207+ na mucosa, duas populações de células dendríticas mielóides (CDm) (ii) CD11b+/CD11c- e (iii) CDm CD11b+/CD11c+ na interface mucosa e submucosa, e (iv) CDp na submucosa. As duas últimas CDMO induzem a produção de IFN-γ e lL-10 por LTCD4+, sugerindo propriedades tolerogênicas das mesmas. No entanto, as CDs alteram seu fenótipo entre a mucosa oral e os linfonodos regionais até onde elas migram[68]. Durante o processo de migração aos linfonodos regionais, as CDMO expressam altos níveis de marcadores de maturação tais como o CD40, CD80, CD86, assim como CD83 e CD208 (DC-LAMP)[69,70].

Há um reconhecimento crescente de que processos inflamatórios podem propiciar o desenvolvimento e progressão tumoral, através de citocinas produzidas por células tumorais e por células da imunidade inata (CDs e macrófagos)[71]. Este processo inflamatório tipicamente falha na estimulação da imunidade mediada por CLs e contribui com a progressão tumoral via supressão ativa da imunidade adaptativa (linfócitos)[72].

1.6.3.1 Células de Langerhans (CLs) e células dendríticas submucosas (CDsub)

As CLs pertencem à família de CDs e são células apresentadoras de antígenos para linfócitos T localizados no epitélio[72]. As CLs são derivadas da medula óssea e representam 2-3% da população celular total de epitélio normal da pele e da mucosa. Estas CDs imaturas expressam S110, CD1a e CD207[65,73]. Estudos mostraram que o número de CDs está aumentado e está associado a eventos de supressão tumoral em pacientes com carcinomas de pulmão, nasofaríngeo, gástrico, esofágico e de mama[74]. Neste contexto, alguns estudos avaliaram a presença de CDs e sua relação com o CECO e DPMs[74]. CDs CD1a+ foram observadas em uma concentração maior na região intratumoral de CECO e

CEC de lábio (em íntima associação com células neoplásicas) ou em localização intraepitelial nas DPMs[74]. Interessantemente, estudos mostram que infecção pelo HPV modula o ambiente imune tumoral. Assim, Nguyen et al.[75] (2016) relataram que o número de CLs (CD1a+) está reduzido no estroma tumoral de pacientes jovens com CECorof HPV+. Kindt et al.[76] (2016) mostraram em seu estudo que houve um maior número de CLs na região intratumoral de CECO do que CECorof e não foram observadas diferenças no estroma tumoral. Goldman et al.[77] (1998) avaliaram a correlação entre recidiva e sobrevida de pacientes com CECO (língua) e a quantidade de CLs no tumor e em áreas adjacentes. Os resultados desse estudo mostraram que pacientes com expressão positiva de CD1a nas CDs adjacentes ao tumor apresentaram maior sobrevida e menor taxa de recidiva. Gallo et al.[78] (1991) avaliaram a expressão de CLs em 88 espécimes de CEC de laringe e observaram que houve uma correlação positiva entre a densidade de CLs e a sobrevida dos pacientes. Além disso, a marcada presença de infiltrado linfoide junto ao tumor também pode ser considerada um bom fator prognóstico para pacientes com CEC de laringe[78]. Recentemente, Jardim et al.[65] (2018) associaram que a depleção de CDs CD1a+ peritumorais é um fator prognóstico independente, relacionando-as com altas taxas de recorrência, metástase linfonodal e pior sobrevida em CECO.

O'Donnell et al.[79] (2007) avaliaram a distribuição de CDim (CD207, CD209), CDm (CD208) e CDp (CD123) em 63 casos de CECO e 8 metástases linfonodais através da expressão imunoistoquímica dos anticorpos contra esses subgrupos de CDs. Os resultados mostraram que CDim estavam presentes no tumor primário; no entanto, raramente encontravam-se infiltrando o tumor. A presença de CDm e CDp foi escassa e geralmente estava associada a pior prognóstico. Esses resultados sugerem que a resposta deficiente das CDs em lesões tumorais parece estar relacionada à função alterada e não a falha no recrutamento dessas células. Portanto, uma estratégia mais eficaz para reestabelecer a função imunológica em resposta ao CEC seria manter o equilíbrio dos fatores secretados no microambiente tumoral, ao invés de restaurar somente uma única população de CDs. Em estudo realizado para avaliar CDsub (FXIIIa+/CD209+) em DPMs, CECO e CEC de lábio inferior, desenvolvido pelo nosso grupo, foi possível identificar um aumento progressivo de CDsub em displasia epitelial de alto grau das DPMs,

seguido pelo CECO e CEC de lábio inferior. Os resultados sugerem uma participação das CDsub na patogênese dessas lesões, possivelmente induzindo tolerância imunológica, mesmo em estágios iniciais da carcinogênese oral e do lábio inferior[80].

1.6.3.2. Células dendríticas plasmocitóides

As CDp compreendem um subgrupo de CDs que produzem grandes quantidades de interferon tipo 1 (IFN-1), classificada como citocina supressora de tumores[79]Donell et al., 2007). De fato, essa liberação de grande quantidade de IFN-1 está associada com uma variedade de agentes, incluindo DNA viral, desempenhando um papel crítico nas respostas imunológicas naturais antivirais iniciais[81-83]. As CDp podem ser avaliadas através dos imunomarcadores CD123 e CD303. Este último se encontra frequentemente expresso em CDp em processo de maturação. Pellicioli et al. (2017) observaram um aumento significativo de CDp CD303+ em CECO, quando comparados com displasia epitelial oral. O'Donell et al.[79] (2007) avaliaram a presença de CDp CD123+ e observaram que houve uma significativa associação com a diminuição da sobrevida de pacientes com CECO e metástase linfonodal. Recentemente, mostrando a importância do papel do sistema imune. Abolhalaj et al.[82] (2018) realizaram um estudo comparativo de CDm (CD11c+) e CDp (CD123+) com CECorof e tecido normal da mesma região, demonstrando que CD123+ teve uma menor expressão em CECorof do que em tecido normal da região, enquanto que a presença de CDm foi significativamente mais alta em CECorof[82].

2 PROPOSIÇÃO

2.1 Objetivo Geral

O desenvolvimento do CECO e CECorof possuem eventos que permitem sua progressão por meio da inibição ou ativação de diferentes mecanismos moleculares, favorecendo a progressão tumoral. Assim, o objetivo do presente estudo está sendo analisar comparativamente, utilizando um amplo painel IQ, suportado por HIS, os diversos eventos celulares mediados por CDs e a oncogênese viral através de associações clinicopatológicas, focando fatores prognósticos e terapêuticos.

2.2 Objetivos Específicos

- Avaliar, por IQ, a expressão dos imunomarcadores de CLs S100, CD1a, CD207 no CECO (n=109) e CECorof (n=126) e estabelecer associações clinicopatológicas.
- Avaliar, por IQ, a expressão dos imunomarcadores de CDsub CD209 e FXIIIA no CECO (n=109) e CECorof (n=126) e estabelecer associações clinicopatológicas.
- Avaliar, por IQ, a expressão dos imunomarcadores de CDp CD123 e CD303 no CECO (n=109) e CECorof (n=126) e estabelecer associações clinicopatológicas.
- Avaliar, por IQ, a expressão dos imunomarcadores de CDm CD83 e CD208 no CECO (n=109) e CECorof (n=126) e estabelecer associações clinicopatológicas
- Avaliar, por HIS, a presença de agentes virais (HPV e VEB) no CECO (n=109) e CECorof (n=126) e estabelecer associações clinicopatológicas.

3 PUBLICAÇÃO*

3.1 Publicação 1

TUMOR-INFILTRATING DENDRITIC CELLS AND ACTIVATION STATUS IN ORAL AND OROPHARYNGEAL SQUAMOUS CELL CARCINOMAS: AN IMMUNOHISTOCHEMICAL AND IN SITU HYBRIDIZATION STUDY

Heitor Albergoni Silveira[1,2], Luciana Yamamoto Almeida[2], Roman Carlos[3], Evânio Vilela Silva[2], Tulio Morandin Ferrisse[2], Andressa Duarte[3], Suziene Caroline Silva Cardoso[3], Xiomara Beatriz Jimenez Polanco[1], Andreia Bufalino[2], Alfredo Ribeiro-Silva[3], Jorge Esquiche León[1]

[1] Oral Pathology, Department of Stomatology, Public Oral Health, and Forensic Dentistry, Ribeirão Preto Dental School (FORP/USP), University of São Paulo, Ribeirão Preto, SP, Brazil.

[2] Oral Medicine, Department of Diagnosis and Surgery, Araraquara Dental School, São Paulo State University (UNESP), Araraquara, São Paulo, Brazil.

[3] Centro Clínico de Cabeza y Cuello, Guatemala City, Guatemala.

[4] Department of Pathology and Forensic Medicine, Ribeirão Preto Medical School (FMRP/USP), University of São Paulo, Ribeirão Preto, SP, Brazil.

Key-Words: Head and neck squamous cell carcinoma; oropharyngeal; oral cavity; dendritic cells; HPV; immunohistochemistry; in situ hybridization.

Funding:

Heitor Albergoni Silveira (2018/12734-2), Luciana Yamamoto Almeida (2016/02713-2) and Jorge Esquiche León (2016/11419-0) have received research Grants from State of São Paulo Research Foundation (FAPESP).

* O artigo segue as normas para publicação no periódico Oral Oncology (ISSN: 1368-8375) para o qual pretende-se submeter.

ABSTRACT

OBJECTIVES: Several studies show a high prevalence of human papillomavirus (HPV) infection in oropharyngeal (OPSCC) than oral cavity (OSCC) squamous cell carcinoma. Considering that tumor cell-dendritic cell (DC) interactions, as well as antiviral immune responses (relevantly plasmacytoid DCs), could play pivotal roles on its pathogenesis, the significance of these findings are unknown.

Materials and Methods: One hundred and nine OSCCs and 126 OPSCCs were assessed through immature (imDC; S100, CD1a, CD207), mature (mDC; CD83 and CD208), submucosal (subDC; FXIIIa and CD209) and plasmacytoid (pDC; CD123 and CD303) DC immunomarkers. Moreover, $p16^{INK4a}$ immunoexpression and in situ hybridization analysis for HPV and Epstein-Barr virus (EBV), were performed.

Results: A significant high HPV prevalence in OPSCC (25%) than OSCC (11%) was observed. Overall, imDC markers, an in lesser number by mDC markers, were significantly more often in OPSCC than OSCC. Differently, OSCC than OPSCC presented a higher number of subDC markers. Both neoplasms presented similar amounts of activated pDCs. HPV-positive than HPV-negative group showed a higher number of DC markers in both neoplasms. Noteworthy, a significant higher number of CD207+ and CD123+ cells were observed in HPV-associated OPSCC than HPV-associated OSCC. All cases were EBV negative. Different from CD123, high expression levels of CD208 was associate with better prognosis in OPSCC.

CONCLUSIONS: Different from OSCC, our results show predominance of imDCs, with immune cell activation profile, in OPSCC. HPV status seems to show association with DC infiltration in both neoplasms, notably suggesting antiviral immune responses in HPV-associated OPSCC.

Key-Words: Head and neck squamous cell carcinoma; oropharyngeal; oral cavity; dendritic cells; HPV; immunohistochemistry; in situ hybridization.

INTRODUCTION

The head and neck squamous cell carcinoma (HNSCC) occupy the sixth position of cancer incidence around the world, and according to current statistics about 600,000 new cases are diagnosed and 350,000 deaths are reported annually [1, 2]. Several studies indicate that there is a variation in incidence worldwide, as well as the habits and customs of each region. The well-defined risk factors for HNSCC are mainly alcohol and tobacco consumption, but in some countries, like USA, Canada and Norway, the association with the human papillomavirus (HPV), specifically the HPV subtype 16, seems to be a well-established risk factor for the oropharyngeal squamous cell carcinoma (OPSCC) pathogenesis. The HPV-associated OPSCC affects most commonly nondrinker, nonsmoker young patients and, relevantly, HPV-positive than HPV-negative OPSCC patients appear to have a better prognosis [3-6]; however, other studies show significant variability depending on the geographic region assessed [7, 8]. Oral cavity squamous cell carcinoma (OSCC) affect an older population, that has smoking and drinking habits. Different from OPSCC, the HPV prevalence in OSCC is low (approximately 10% to 25% of cases) [9, 10].

The dendritic cells (DCs) are considered potent antigen-presenting cells (APCs), being able to initiate and modulate the host immune response, as well as participate in the antitumor response. After antigen capture, immature CDs (imCDs) migrate to a lymphoid organ (e.g., lymph nodes) and interact with T lymphocytes to be able to mature and then initiate immune responses [11, 12]. DCs are also considered to be the best vehicle for the delivery of tumor-specific antigens in cancer immunotherapy [13-15]. Cancer immunization studies suggest that the immune system, at an early stage of cell dysplasia or tumorigenesis, may have an ability to eliminate cells that have suffered DNA damage, which can evolve into malignancy [12, 16]. Other studies focusing DCs in cancer have attracted much interest because of their association to be involved in immunosurveillance with the antitumor response [17]. The interactions between DCs with their environment occur through molecules located in the cell membrane, so the phenotype of DCs is important for their identification, as well as understanding their mechanism of action [18]. The DCs can be classified as myeloid (myDCs) and plasmacytoid (pDCs), including also its activation status or maturation state [19].

Several studies have assessed the presence of DC subtypes in lung, esophagus, gastrointestinal tract and breast carcinoma [11, 20-22]. In the head and neck region, DC subtypes were evaluated in the lower lip SCC and OSCC, including potentially malignant disorders such as actinic cheilitis, oral leukoplakia, oral lichen planus and oral submucosal fibrosis, through S100 [23-26], CD1a [4, 11, 12, 17, 25-32], CD207 [12, 29, 33], CD123 [33, 34], CD303 [28, 29], CD83 [11, 17, 27, 28, 32], CD208 [36, 36], and CD209 [33] markers. Noteworthy, to date, only three studies [4, 37, 38] have assessed DC subtypes in OPSSC. To the best of our knowledge, no comparative study considering DC subtypes between OSCC and OPSCC has been conducted to date.

Therefore, the objective of the current study was to perform a comparative immunohistochemical (IHC) characterization of DC subtypes in OSCC and OPSCC, in correlation with HPV status, aiming at a better understanding of the innate immune system participation in the pathogenesis of these tumors.

MATERIAL AND METHODS

Patients and samples

The study was approved by the Human Research Ethics Committee from Ribeirão Preto Medical School, University of São Paulo (FMRP/USP) CAAE: #99417018.4.0000.5440. In this study, 126 OPSCCs and 109 OSCCs were assessed. Formalin-fixed, paraffin-embedded tissue blocks from surgical specimens were retrieved from the files at the Laboratory of Anatomical Pathology, Ribeirão Preto Medical School, University of São Paulo (FMRP/USP). A retrospective study was conducted, the clinical data for all cases were obtained from medical records, including the age, gender, ethnicity, clinical stage, TNM, smoking and alcohol consumption status, follow-up duration, outcome and survival status.

Histopathological evaluation

Two expert pathologists (A.R.S and J.E.L) reviewed the original 5-µm histological tissue sections, mounted on a glass slide, and stained with hematoxylin and eosin (H&E). All cases were diagnosed according to the criteria proposed by World Health Organization (WHO, 2017) of head and neck tumors [39].

Construction of Tissue Microarray (TMA)

After morphological review of all the cases, a more representative area of the tumor was selected and later marked. The slides were placed on the original paraffin tissue block to determine the corresponding area to be used in the TMA construction. The TMA blocks were constructed using a manual tissue matrix (Sakura Co., Tokyo, Japan), each containing cylindrical cores (2.0-mm diameter each) [40, 41]. All antibodies (except $p16^{INK4a}$) and probes were assessed on TMA slides with cores in duplicate. A conclusive result was obtained, if there was no difference between duplicate TMAs. A conclusive result was obtained, if there was no difference between duplicate TMAs.

Immunohistochemical (IHC) analysis

The TMA blocks were cut to a thickness of three-µm, which were placed on glass slides properly coated with organosilane (Sigma-Aldrich, St Louis, MO, USA). The slides were dewaxed with xylene and rehydrated in descending ethanol solutions (absolute, 90%, 80% and 70%). The slides were submitted to the IHC technique by using the streptavidin-biotin-peroxidase method (K0690; Universal, Dako LSAB®+ Kit, Peroxidase, Carpinteria, CA, USA) to evaluate individual primary antibodies, according to the manufacturer's protocol (Table 1). Negative control specimens included replacing the primary antibody with isotype-specific serum. The samples were counterstained with Carazzi's hematoxylin.

In situ hybridization (ISH) for HPV and EBV (EBER)

To determine HPV or Esptein-Barr virus (EBV) infection, all cases were assessed by ISH. Epstein–Barr encoding region (EBER) ISH, was performed with fluorescein isothiocyanate (FITC)-conjugated EBER1/2 PNA-probes (DAKO, code Y5200). Signal amplification was developed with anti-FITC alkaline phosphatase-conjugated antibody and 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate substrate combined with levamisole (DAKO, code K 5201). In situ HPV DNA detection was performed with biotinylated probes Y1404 WideSpectrum (WS) including HPV genotypes 6, 11, 16, 18, 31, 33, 35, 39, 45, 51 and 52; Y1411 HPV types 6/11 DNA Probe Mix; and Y1443 GenPoint HPV, biotinylated DNA Probe targeting sequences of "high-risk" HPV genotypes 16, 18 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Signal amplification was developed with K620 (GenPoint, Dako, Carpinteria, CA, USA).

Scoring

All the slides were firstly evaluated at ×100 magnification, after this, five representative areas were selected and registered in a microscopic field at ×400 magnification, through an optical microscope (Leica DM500) adapted to a high-resolution camera (Leica IC50). All positive cells in the captured image were quantified and a total cylinder mean was obtained, using the Image J software (National Institutes of Health, Bethesda, MD).

Statistical analysis

To evaluate the normality distribution, the skewness and kurtosis analysis and Shapiro-Wilk test, were used. To assess homoscedasticity, the Box's test for equivalence of covariance matrices and Levene's test for equality of variances, were applied. Moreover, the one-way MANOVA analysis, Student's t test, Mann-Whitney's test, Test G and Kruskal-Wallis test, were used when indicated. A p-value of <0.05 was regarded to be statistically significant. Statistical analysis was conducted by IBM SPSS Statistics 20.0 and graphics image with GraphPad Prism 6.0

RESULTS

The clinicopathological features are shown in the **Table 02**. The HPV profile, location, morphological classification and $p16^{INK4a}$ status are detailed in the T**able 03.**

Both OSCC and OPSCC commonly affected male patients, with a mean age of 60 years. OPSCC than OSCC present higher rates of smoking and drinking habits ($p<0.0001$). For TNM classification, without tumor size difference, N0-N1 was more frequent in OSCC and N2-N3 in OPSCC. OSCC than OPSCC presented higher recurrence rate (40.9% vs 33.7%); however, metastasis was more often in OPSCC ($p=0.0271$) (Table 02).

A significant high HPV prevalence in OPSCC (32 cases, 25%) than OSCC (12 cases, 11%) was observed. In OPSCC and OSCC, 19 and 7 were high-risk HPV (HRHPV) only, 6 and 3 low-risk HPV (LRHPV) only, 3 and 2 HRHPV/LRHPV, and 4 and 0 WS-HPV, respectively. In the OSCC, 11/12 (92%) cases, and in the OPSCC, 29/32 (90%) cases, were $p16^{INK4a}$ immunopositive (Table 03). All cases were EBER negative.

The results of the DC infiltration are shown in the **Tables 04-07** and **Figures 01-04.** Overall, Langerhans cell markers (or imDcs) were more frequently observed,

followed by subDC markers and, in lesser number, by roughly similar amounts of mDC and pDC markers, in both OSCC and OPSCC.

Regarding DC infiltration in OSCC and OPSCC, Langerhans cell markers were significantly more often in OPSCC. Differently, OSCC presented a higher number of subDC markers (XIIIa, p=0.071; CD209, p<0.0001). The mDC markers were more frequent in OPSCC (CD83, p=0.123; CD208, p=0.037), whereas pDC markers were observed in similar amounts (Table 04).

The DC infiltration in OSCC considering the HPV status is shown in the Table 05. Although a higher number of DC markers was observed in HPV-positive than HPV-negative group, there were no statistical significant differences. Relevantly, in the HPV-negative group, CD208+ and CD303+ cells were not detected.

The DC infiltration in OPSCC considering the HPV status is shown in the Table 06. Similar with OSCC, the HPV-positive than HPV-negative group exhibited a higher number of DCs for all but CD209 markers. Statistically significant differences were found only for CD1a (p=0.020) and CD207 (p=0.018); followed by CD208 (p=0.078) marker.

The DC density in OSCC and OPSCC considering only the HPV+ groups, is shown in the Table 07. Except for subDC markers and with similar amount of CD303+ cells, all other markers were more frequently observed in OPSCC. Statistically significant differences were found only for CD207 (p=0.014) and CD123 (p=0.046); followed by S100 (p=0.061) and CD1a (p=0.077).

Regarding the DC marker expression in correlation with the clinicopathological variables, the low expression levels of CD208 showed significant association with recurrence and metastasis, whereas the high expression levels of CD123 showed significant association with low overall survival rate, in OPSCC patients.

DISCUSSION

In the current study, the HPV prevalence was 13% for OSCC and 25% for OPSCC. All but 04 HPV-associated neoplasms exhibited immunopositivity for $p16^{INK4a}$. The literature, data show HPV association between 10% to 25% of the OSCC cases [9, 10, 42]; however, for OPSCC this prevalence is variable. In fact, different from Central and South America (14.9%), North America (54.7%), Asia (45%), Oceania (42.1%) and Europe (36.2%), show high HPV prevalence in OPSCC cases. Data from Brazil indicate that the prevalence of HPV-associated OPSCC varies between 5% to 25% [5, 7, 8, 43-45], such as found in the current study.

Overall, several studies indicate that HPV-associated OPSCC are often detected in nondrinker, nonsmoker young patients, and associated with a better prognosis [7, 46, 47]. In the current study, we have observed similar clinicopathological features when comparing gender and age between OSCC and OPSCC patients, however, this latter presented higher rates of smoking and drinking habits. Moreover, recurrence and metastasis exhibited higher rates in OSCC and OPSCC, respectively (Table 02). These findings suggest that the prognostic factors in OSCC and OPSCC in a Brazilian population should be carefully assessed.

Such as above commented, in the current study, the OPSCC than OSCC showed higher prevalence of HPV infection. In this context, the analysis of the tumor cell-DC interactions, the maturation status of DCs, as well as the antitumor and antiviral immune responses [12, 17], is essential for a better understanding of the pathogenesis of these tumors.

The DCs are considered potent antigen-presenting cells (APCs), being able to initiate and modulate the host immune response, as well as participate in the antitumor and antiviral responses [11, 12, 14]. Langerhans cells (LCs) are bone marrow-derived cells, mainly located in the epithelium of the skin and mucosa, representing approximately 2.5% of all cells in these locations [32]. LCs are a distinct imDC subtype (exhibiting the S100+/CD1a+/CD207+ immunoprfile), which after antigen capture and subsequent displacement to regional lymph nodes, are able to differentiate into mDCs. The mDCs define a DC subtype with activation status, characterized by expression of the CD83 and CD208 markers [19].

Several studies have evaluated the expression of imDC markers in OSCC, lower lip SCC, as well as in potentially malignant disorders [11, 12, 17, 23-36]. However, only two studies have assessed imDC markers in OPSCC cases, which were included within a large case series of HNSCC [4, 37]. In the current study, for the first time, we present a detailed and comparative clinicopathological analysis regarding the imDC immunoexpression, in correlation with HPV status, in a large case series of OSCC and OPSCC. Our results show that imDCs were significantly more often in OPSCC than OSCC. Moreover, the HPV-positive than HPV-negative group exhibited a higher number of imDCs in both OSCC and OPSCC, with statistically significant differences only in OPSCC. These findings, unlike from

previous findings [4], suggest that the HPV infection could be modulating the presence of imDCs in these neoplasms. Moreover, although some studies suggest that the presence of infiltrating DCs in tumors may indicate the host immune response, reflecting a better prognosis [30, 32, 48], our results reinforce previous findings which suggest that an ineffective DC response to OSCC cells is a failure of DC function (ie, impaired maturation) rather than recruitment [33], indicating that further strategies should be adopted to restore the antitumor immune response.

The interstitial or dermal DCs are commonly located in the upper dermis, being capable and efficient in alerting the immune system. When located in mucosal surface, the subDC denomination seems to be more appropriate [49, 50]. This DC subtype is characterized by the expression of major histocompatibility complex (MHC) class II molecules, the scavenger receptor CD36, the coagulation factor XIIIa (FXIIIa) and CD209 (DC-SIGN). The FXIIIa, a coagulation transglutaminase, is a marker for macrophages and dermal/submucosal DCs reported to be bone marrow-derived, with phagocytic and antigen-presenting properties. The CD209 is a C-type lectin receptor present on the surface of both macrophages and DCs in several tissues, including skin, mucosa and lymph nodes [49, 50]. However, as they usually lack co-stimulatory molecules (immature state), this is likely to have the effect of suppressing rather than inducing an immune response [51, 52]. To date, the role of subDCs in the pathogenesis of HNSCC is not yet well understood.

To the best of our knowledge, only one study has assessed the CD209 marker in OSCC, which evidenced marked presence in the tumor stroma, suggesting immunotolerance properties [33]. In the current study, OSCC presented a higher number of subDC markers (XIIIa, $p=0.071$; CD209, $p<0.0001$) than OPSCC. Notably, the amount of imDCs and subDCs in OSCC were similar. Regarding the low HPV prevalence in OSCC than OPSCC, our results suggest participation of the subDCs in mechanisms of tumor immunity, regardless of HPV presence.

The DC maturation status is essential for effective antigen presentation and initiation of the primary immune response. In fact, imDCs phagocytize pathogens and toxic proteins for presentation on MHC class II molecules to naïve T cells. During activation, the imDCs become mature. Maturation begins when the DCs start

migrating from peripheral tissues to regional lymph nodes. During antigen presentation, the DCs upregulate costimulatory receptor molecules, such as CD80, CD83, CD86, CD208, and CD40. These activated DC receptor molecules bind receptors on the Th0 cell membrane, resulting in T cell differentiation, with preferentially antitumor response [32, 53].

Several studies have evaluated mDCs in OSCC, lower lip SCC and potentially malignant disorders, through CD83 and CD208 markers [11, 17, 27, 28, 32, 35, 36]. Ni et al. (2014) [35] observed an increased imDC infiltration in OSCC, but their results showed no correlation with the patient survival. Unfortunately, the mDC profile in OPSCC is poorly understood [37]. In the current study, the expression of mDC markers was higher in OPSCC than OSCC (CD83, p=0.123; CD208, p=0.037). Moreover, without statistical differences, the expression of mDC markers was higher in HPV-positive than HPV-negative group in both OSCC and OPSCC. These findings suggest a dynamic DC maturation status in these neoplasms, which could be modulated by the presence of HPV, and probably also by tumor immune mechanisms. In spite of this, which appears to be happening at low levels, it is evident the difference when comparing imDC and mDC markers, reflecting perhaps local defects in the activation mechanisms of the immune system, such as previously proposed [33], and/or alternatively, due to the migration of the mDCs to regional lymph nodes [28]. Moreover, in the current OPSCC series, the low expression of CD208 showed significant association with recurrence and metastasis. These findings reinforce the prognostic impact of DC maturation status, considering the reciprocal relationship between cancer cells and its microenvironment.

The pDCs are highly efficient in processing intracellular viral or self DNA and/or RNA molecules, notably through Toll-like receptors, producing rapidly large amounts of type I and III interferons. Thus, pDCs play an important role in antiviral immunity and autoimmune disease [34, 55, 56]. The pDCs express the CD303 (BDCA2), CD304 (BDCA4), CD123 (IL-3R), and CD45RA markers (Dzionek, 2001). I It has been suggested that the CD123+ cell infiltration into HNSCC [33, 55], as well as in melanoma and lung and ovarian cancer [57, 58], is associated with a worse prognosis. Different from CD123, which is constitutively expressed, the simultaneous CD303 expression on pDCs indicates an immature profile [54, 55]. In this context, some studies have evaluated the CD123 and CD303 expression in OSCC and potentially malignant disorders [28, 29, 33, 34]; however, the pDC maturation status

was not emphasized. In spite of this, these works highlight the pDC participation in the oral carcinogenesis and its association with an adverse outcome in OSCC.

In the current study, without statistically significant differences, a higher number of pDCs in HPV-positive than HPV-negative group in both OSCC and OPSCC was observed. Noteworthy, when assessing the pDC density only between HPV-associated OSCC and OPSCC cases, a significant higher number of CD123+ cells in OPSCC was visualized. Moreover, we have detected a significant higher number of CD123+ than CD303+ cells in both OSCC and OPSCC, indicating a maturation process for pDC population [54, 55]. Taken together, and considering the high HPV prevalence in OPSCC, our results suggest that antiviral immune responses in HPV-associated OPSCC should also be considered, Moreover, similar with previous studies [28, 29, 33, 34], the high expression levels of CD123 showed significant association with low overall survival rate in OPSCC patients. Thus, pDC markers may become relevant prognostic factors for these neoplasms.

In summary, different from OSCC, our results show predominance of imDCs, with immune cell activation profile, in OPSCC. HPV status seems to show association with DC infiltration in both neoplasms, notably suggesting antiviral immune responses in HPV-associated OPSCC. Further studies focusing on molecular aspects to evaluate the functional status of DC subtypes, as well as their activation mechanisms on the tumor microenvironment, will probably offer a more understanding for immunotherapy strategies in the treatment of HPV-associated and HPV-unassociated HNSCC patients.

REFERENCES

[1] Pestana RC, Becnel M, Rubin ML, Torman DK, Crespo J, Phan J, et al. Response rates and survival to systemic therapy after immune checkpoint inhibitor failure in

recurrent/metastatic head and neck squamous cell carcinoma. Oral Oncol. 2019; 18; 101: 104523. [Epub ahead of print]

[2] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancerstatistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394-424.

[3] Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol. 2011 Nov 10;29:4294-4301.

[4] Kindt N, Descamps G, Seminerio I, Bellier J, Lechien JR, Pottier C, et al. Langerhans cell number is a strong and independent prognostic factor for head and neck squamous cell carcinomas. Oral Oncol. 2016;62:1-10.

[5] Ndiaye C, Mena M, Alemany L, Arbyn M, Castellsagué X, Laporte L, et al. HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. Lancet Oncol. 2014;15:1319-1331.

[6] Fossum GH, Lie AK, Jebsen P, Sandlie LE, Mork J. Human papillomavirus in oropharyngeal squamous cell carcinoma in South-Eastern Norway: prevalence, genotype, and survival. Eur Arch Otorhinolaryngol. 2017;274:4003-4010.

[7] De Cicco R, de Melo Menezes R, Nicolau UR, Pinto CAL, Villa LL, Kowalski LP. Impact of human papillomavirus status on survival and recurrence in a geographic region with a low prevalence of HPV-related cancer: A retrospective cohort study. Head Neck. 2020;42:93-102.

[8] Nopmaneepaisarn T, Tangjaturonrasme N, Rawangban W, Vinayanuwattikun C, Keelawat S, Bychkov A. Low prevalence of p16-positive HPV-related head-neck cancers in Thailand: tertiary referral center experience. BMC Cancer. 2019 Nov 6;19:1050

[9] Hübbers CU, Akgül B. HPV and cancer of the oral cavity. Virulence. 2015;6:244-248.

[10] Syrjänen S, Lodi G, von Bültzingslöwen I, Aliko A, Arduino P, Campisi G, et al. Human papillomaviruses in oral carcinoma and oral potentially malignant disorders: a systematic review. Oral Dis. 2011;17 Suppl 1:58-72.

[11] Costa NL, Gonçalves AS, Martins AF, Arantes DA, Silva TA, Batista AC. Characterization of dendritic cells in lip and oral cavity squamous cell carcinoma. J Oral Pathol Med. 2016;45:418-424.

[12] Öhman J, Magnusson B, Telemo E, Jontell M, Hasséus B. Langerhans cells and T cells sense cell dysplasia in oral leukoplakias and oral squamous cell carcinomas--evidence for immunosurveillance. Scand J Immunol. 2012;76:39-48.

[13] Mayordomo JI, Zorina T, Storkus WJ, Zitvogel L, Celluzzi C, Falo LD, et al. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. Nat Med. 1995;1:1297-1302.

[14] Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol. 2000;18:767-811.

[15] Kacani L, Wurm M, Schwentner I, Andrle J, Schennach H, Sprinzl GM. Maturation of dendritic cells in the presence of living, apoptotic and necrotic tumour cells derived from squamous cell carcinoma of head and neck. Oral Oncol. 2005;4:17-24.

[16] Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 2002;3:991–998.

[17] Gomes JO, de Vasconcelos Carvalho M, Fonseca FP, Gondak RO, Lopes MA, Vargas PA. CD1a+ and CD83+ Langerhans cells are reduced in lower lip squamous cell carcinoma. J Oral Pathol Med. 2016;45:433-439.

[8] Clark GJ, Silveira PA, Hogarth PM, Hart DNJ. The cell surface phenotype of human dendritic cells. Semin Cell Dev Biol. 2019;86:3-14.

[19] da Motta RJ, Tirapelli C, Juns da Silva R, Villafuerte KR, Almeida LY, Ribeiro-Silva A, et al. Immature, but Not Mature, Dendritic Cells Are More Often Present in Aggressive Periodontitis Than Chronic Periodontitis: An Immunohistochemical Study. J Periodontol. 2016;87:1499-1507.

[20] Zeid NA, Muller HK. S-100 positive dendritic cells in human lung tumors associated with cell differentiation and enhanced survival. Pathology 1993; 25:338-343.

[21] Tsujitani S, Kakeji Y, Watanabe A, Kohnoe S, Maehara Y, Sugimachi K. Infiltration of dendritic cells in relation to tumor invasion and lymph node metastasis in human gastric cancer. Cancer 1990;66:2012–2016.

[22] Matsuda H, Mori M, Tsujitani S, Ohno S, Kuwano H, Sugimachi K. Immunohistochemical evaluation of squamous cell carcinoma antigen and S-100 protein-positive cells in human malignant esophageal tissues. Cancer. 1990;65:2261-2265.

[23] Maloth AK, Dorankula SP, Pasupula AP, Thokala MR, Muddana K, Ramavath R. A Comparative Immunohistochemical Analysis of Langerhans Cells in Oral Mucosa, Oral Lichen Planus and Oral Squamous Cell Carcinoma. J Clin Diagn Res. 2015;9:ZC76-9.

[24] Pereira KM, Soares RC, Oliveira MC, Pinto LP, Costa Ade L. Immunohistochemical staining of Langerhans cells in HPV-positive and HPV-negative cases of oral squamous cells carcinoma. J Appl Oral Sci. 2011;19:378-383.

[25] Hilly O, Strenov Y, Rath-Wolfson L, Hod R, Shkedy Y, Mizrachi A, Koren R, et al. The predictive value of dendritic cells in early squamous cell carcinoma of the tongue. Pathol Res Pract. 2016;212:1138-1143.

[26] Goldman SA, Baker E, Weyant RJ, Clarke MR, Myers JN, Lotze MT. Peritumoral CD1a positive dendritic cells are associated with improved survival in patients with tongue carcinoma. Arch. Otolaryngol. Head Neck Surg. 1998;124;641–646.

[27] Souto GR, Matias MDP, Nunes LFM, Ferreira RC, Mesquita RA. Mature dendritic cell density is affected by smoking habit, lesion size, and epithelial dysplasia in oral leukoplakia samples. Arch Oral Biol. 2018;95:51-57.

[28] Pellicioli ACA, Bingle L, Farthing P, Lopes MA, Martins MD, Vargas PA. Immunosurveillance profile of oral squamous cell carcinoma and oral epithelial dysplasia through dendritic and T-cell analysis. J Oral Pathol Med. 2017;46:928-933.

[29] Silva LC, Fonseca FP, Almeida OP, Mariz BA, Lopes MA, Radhakrishnan R, et al. CD1a+ and CD207+ cells are reduced in oral submucous fibrosis and oral squamous cell carcinoma. Med Oral Patol Oral Cir Bucal. 2019. pii: 23177.

[30] Upadhyay J, Rao NN, Upadhyay RB. A comparative analysis of langerhans cell in oral epithelial dysplasia and oral squamous cell carcinoma using antibody CD-1a. J Cancer Res Ther. 2012;8:591-597.

[31] Ni YH, Zhang XX, Lu ZY, Huang XF, Wang ZY, Yang Y, et al. Tumor-Infiltrating CD1a(+) DCs and CD8(+)/FoxP3(+) Ratios Served as Predictors for Clinical Outcomes in Tongue Squamous Cell Carcinoma Patients. Pathol Oncol Res. 2019; 12. [Epub ahead of print]

[32] Jardim JF, Gondak R, Galvis MM, Pinto CAL, Kowalski LP. A decreased peritumoral CD1a+ cell number predicts a worse prognosis in oral squamous cell carcinoma. Histopathology. 2018;72:905-913.

[33] O'Donnell RK, Mick R, Feldman M, Hino S, Wang Y, Brose MS, et al. Distribution of dendritic cell subtypes in primary oral squamous cell carcinoma is inconsistent with a functional response. Cancer Lett. 2007;255:145-152.

[34] Han N, Zhang Z, Liu S, Ow A, Ruan M, Yang W, et al. Increased tumor-infiltrating plasmacytoid dendritic cells predicts poor prognosis in oral squamous cell carcinoma. Arch Oral Biol. 2017; 78:129-134.

[35] Ni YH, Huang XF, Ding L, Wang ZY, Hu QG, Hou YY. Accumulation of $CD208^{+}$ mature dendritic cells does not correlate with survival time in oral squamous cell carcinoma patients. J Oral Maxillofac Surg. 2014;72:2178-2185.

[36] Wirsing AM, Ervik IK, Seppola M, Uhlin-Hansen L, Steigen SE, Hadler-Olsen E. Presence of high-endothelial venules correlates with a favorable immune microenvironment in oral squamous cell carcinoma. Mod Pathol. 2018;31:910-922.

[37] Abolhalaj M, Askmyr D, Sakellariou CA, Lundberg K, Greiff L, Lindstedt M. Profiling dendritic cell subsets in head and neck squamous cell tonsillar cancer and benign tonsils. Sci Rep. 2018;8:8030.

[38] Sakakura K, Chikamatsu K, Takahashi K, Whiteside TL, Furuya N. Maturation of circulating dendritic cells and imbalance of T-cell subsets in patients with squamous cell carcinoma of the head and neck. Cancer Immunol Immunother. 2006;55:151-159.

[39] El Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ. Classification of Head and Neck Tumours. 4th ed. Lyon, France: IARC Press; 2017.

[40] Radhakrishnan R, Solomon M, Satyamoorthy K, Martin LE, Lingen MW. Tissue microarray - a high-throughput molecular analysis in head and neck cancer. J Oral Pathol Med. 2008;37:166-176.

[41] Neves-Silva R, Almeida LY, Silveira HA, Colturato CBN, Duarte A, Ferrisse TM, et al. SMARCB1 (INI-1) and NUT immunoexpression in a large series of head and neck carcinomas in a Brazilian reference center. Head Neck. 2019 Nov 15. [Epub ahead of print]

[42] Anaya-Saavedra G, Ramírez-Amador V, Irigoyen-Camacho ME, García-Cuellar CM, Guido-Jiménez M, et al. High association of human papillomavirus infection with oral cancer: a case-control study. Arch Med Res. 2008;39:189-197.

[43] Ribeiro KB, Levi JE, Pawlita M, Koifman S, Matos E, Eluf-Neto J, et al. Low human papillomavirus prevalence in head and neck cancer: results from two large case-control studies in high-incidence regions. Int J Epidemiol. 2011;40:489-502.

[44] Li W, Thompson CH, Xin D, Cossart YE, O'Brien CJ, McNeil EB, et al. Absence of human papillomavirus in tonsillar squamous cell carcinomas from Chinese patients. Am J Pathol. 2003;163:2185-2189.

[45] Matos LL, Miranda GA, Cernea CR. Prevalence of oral and oropharyngeal human papillomavirus infection in Brazilian population studies: a systematic review. Braz J Otorhinolaryngol. 2015;81:554-567.

[46] Barros-Filho MC, Reis-Rosa LA, Hatakeyama M, Marchi FA, Chulam T, Scapulatempo-Neto C, et al. Oncogenic drivers in 11q13 associated with prognosis and response to therapy in advanced oropharyngeal carcinomas. Oral Oncol. 2018;83:81-90.

[47] Thompson LDR, Burchette R, Iganej S, Bhattasali O. Oropharyngeal Squamous Cell Carcinoma in 390 Patients: Analysis of Clinical and Histological Criteria Which Significantly Impact Outcome. Head Neck Pathol. 2019;18. [Epub ahead of print]

[48] Gallo O, Libonati GA, Gallina E, Fini-Storchi O, Giannini A, Urso C, Bondi R. Langerhans cells related to prognosis in patients with laryngeal carcinoma. Arch. Otolaryngol. Head Neck Surg. 1991;117;1007–1010.

[49] Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, et al. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell. 2000;100:575-585.

[50] Valladeau J, Saeland S. Cutaneous dendritic cells. Semin Immunol. 2005;17:273-83.

[51] Song Y, Sakamoto F, Ito M. Characterization of factor XIIIa+ dendritic cells in dermatofibroma: Immunohistochemical, electron and immunoelectron microscopical observations. J Dermatol Sci. 2005;39:89-96.

[52] Polak ME, Johnson P, Di Palma S, Higgins B, Hurren J, Borthwick NJ, et al. Presence and maturity of dendritic cells in melanoma lymph node metastases. J Pathol. 2005;207:83-90.

[53] Mbongue JC, Nieves HA, Torrez TW, Langridge WH. The Role of Dendritic Cel Maturation in the Induction of Insulin-Dependent Diabetes Mellitus. Front Immunol. 2017;8:327.

[54] Dzionek A, Sohma Y, Nagafune J, Cella M, Colonna M, Facchetti F, et al. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. J Exp Med. 2001;194:1823-1834.

[55] Yang LL, Mao L, Wu H, Chen L, Deng WW, Xiao Y,et al. pDC depletion induced by CD317 blockade drives the antitumor immune response in head and neck squamous cell carcinoma. Oral Oncol. 2019 Sep;96:131-139. [Epub ahead of print]

[56] Lou Y, Liu C, Kim GJ, Liu YJ, Hwu P, Wang G. Plasmacytoid dendritic cells synergize with myeloid dendritic cells in the induction of antigen-specific antitumor immune responses. J Immunol. 2007;178:1534-41.

[57] Jensen TO, Schmidt H, Møller HJ, Donskov F, Høyer M, Sjoegren P, et al. Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma. Cancer. 2012;118:2476-2485.

[57] Labidi-Galy SI, Treilleux I, Goddard-Leon S, Combes JD, Blay JY, Ray-Coquard I, et al. Plasmacytoid dendritic cells infiltrating ovarian cancer are associated with poor prognosis. Oncoimmunology. 2012;1:380-382.

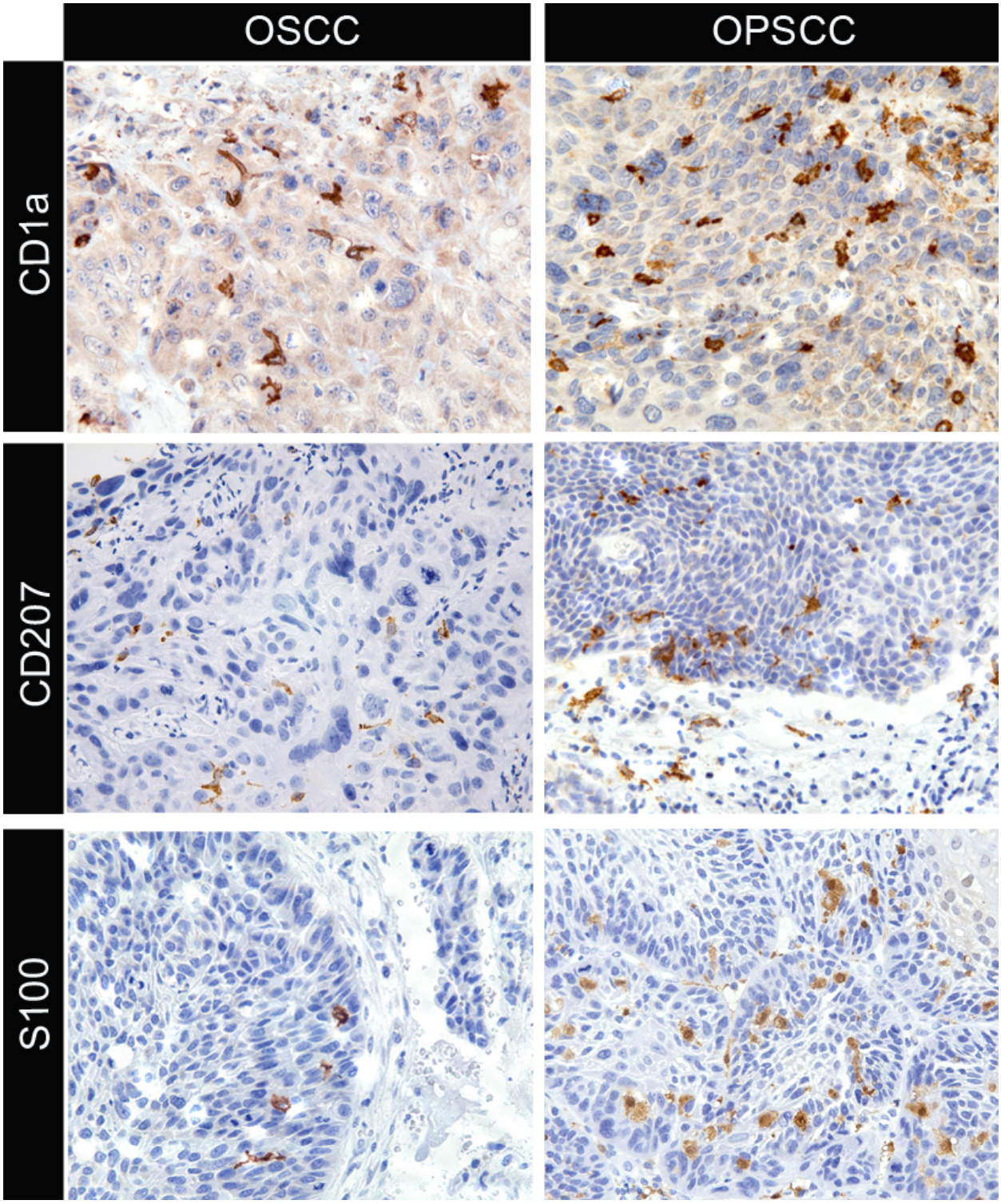

Figure 1: Immunohistochemical analysis of imDCs (CD1a, CD207 and S100) in oral cavity squamous cel carcinoma (OSCC) and oropharyngeal squamous cell carcinoma (OPSCC).

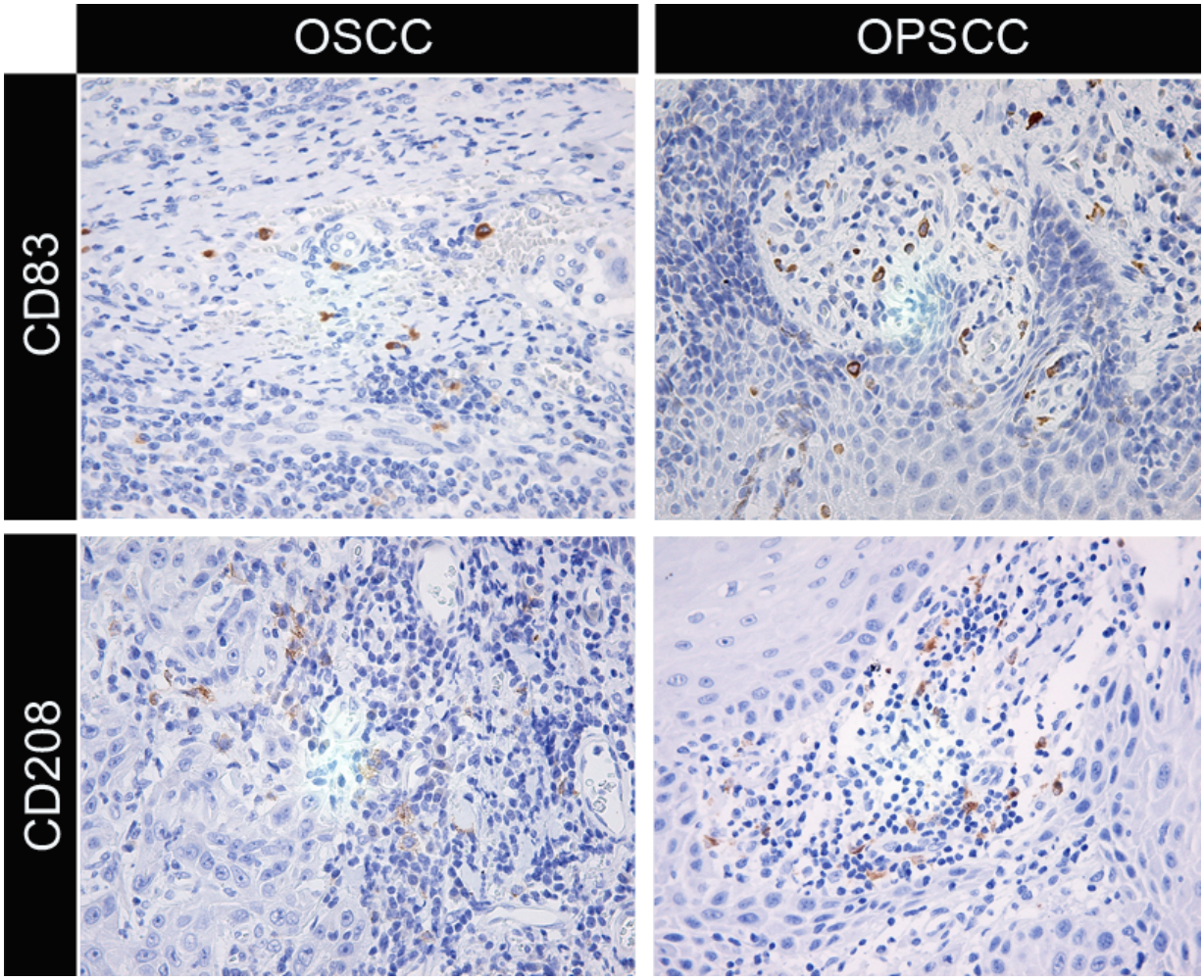

Figure 2: Immunohistochemical analysis of madure dendritics cells (mDCs) (D83 and CD208) in oral cavity squamous cel carcinoma (OSCC) and oropharyngeal squamous cell carcinoma (OPSCC).

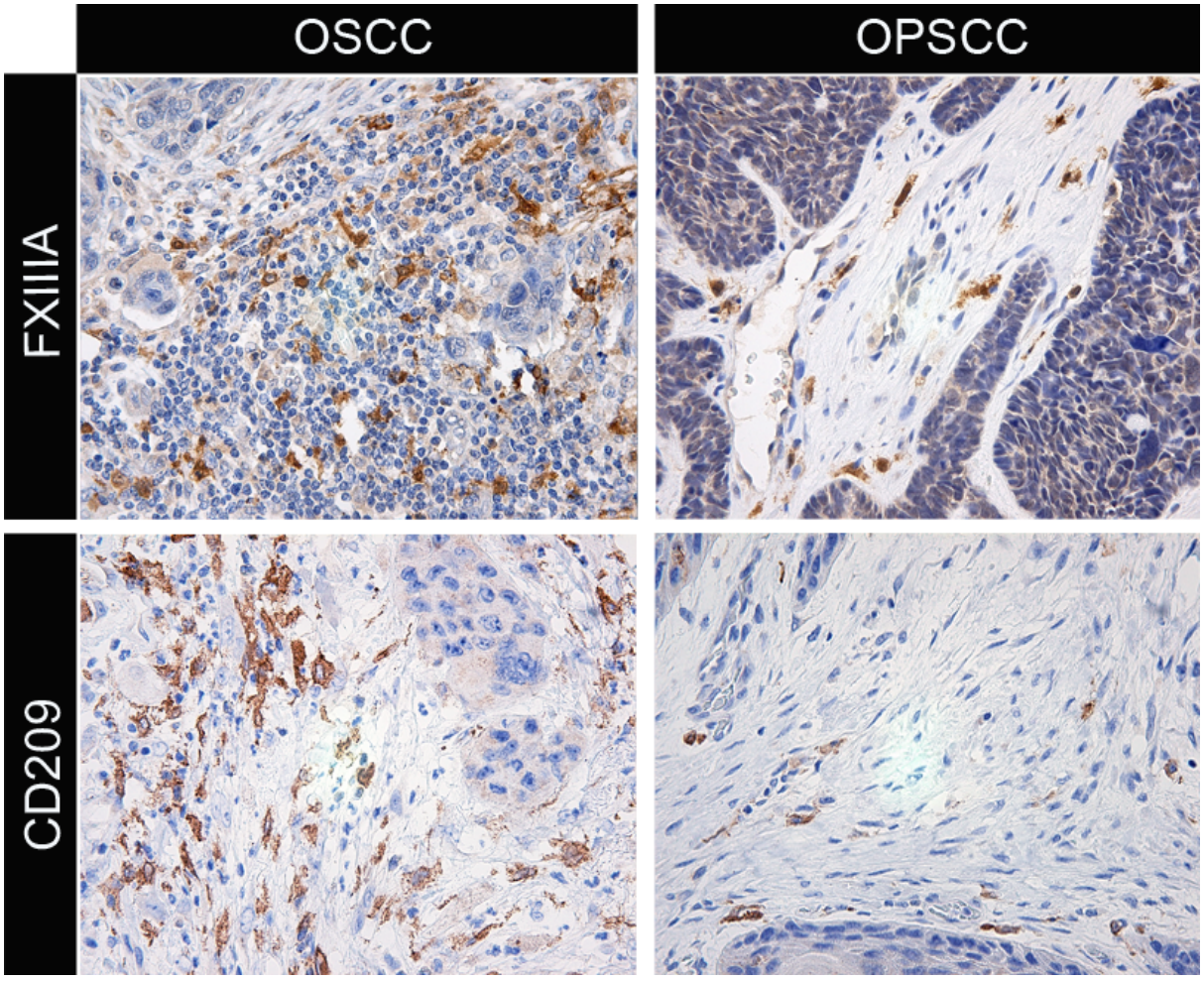

Figure 3: Immunohistochemical analysis of submucosal dendritics cells (subDCs) (FXIIIa and CD209) in oral cavity squamous cel carcinoma (OSCC) and oropharyngeal squamous cell carcinoma (OPSCC).

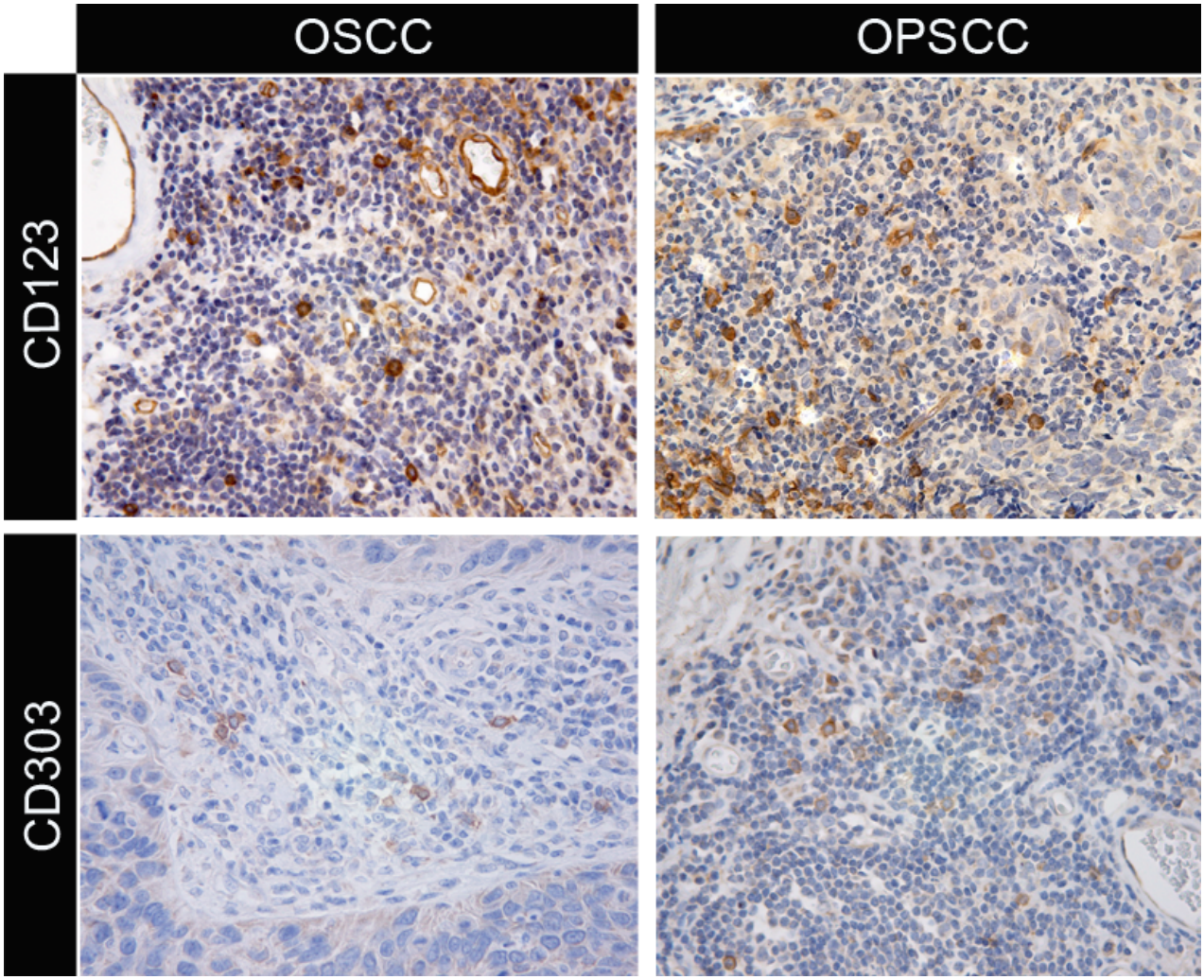

Figure 4: Immunohistochemical analysis of plasmocytoid dendritics cells (pDCs) (CD123 and CD303) in oral cavity squamous cel carcinoma (OSCC) and oropharyngeal squamous cell carcinoma (OPSCC).

Table 01. List of primary antibodies used in the current study.

Antibody	Source	Clone	Dilution	Antigen retrieval	Positive stained cells	Tissue Control
CD1a	DakoCytomation, Glostrup, Denmark	010	1:400	S2367 (DAKO) pH9	Langerhans cells (iDCs)	Palatine tonsil
CD207	Monosan, Uden, the Netherlands	12D6	1:200	S2367 (DAKO) pH9	Langerhans cells	Palatine tonsil
S100	Leica Biosystems Newcastle, UK	Polyclonal	1:3.000	S2367 (DAKO) pH9	Langerhans cells	Palatine tonsil
CD83	Leica Biosystems Newcastle, UK	1H4b	1:100	Citrate buffer pH6	Mature dendritic cells	Palatine tonsil
CD208	Dendritics, Lyon, France	1010E.101	1:500	Citrate buffer pH6	Mature dendritic cells	Palatine tonsil
FXIIIa	Leica Biosystems Newcastle, UK	E980.1	1:100	Citrate buffer pH6	Submucosal dendritic cells	Palatine tonsil
CD209	Epitomics, California, USA	EPR5588	1:5000	S2367 (DAKO) pH9	Submucosal dendritic cells	Palatine tonsil
CD123	Leica Biosystems Newcastle, UK	BR4MS	1:200	S2367 (DAKO) pH9	Plasmocytoid dendritic cells	Palatine tonsil
CD303	Dendritics, Lyon, France	124B3.13	1:500	S2367 (DAKO) pH9	Plasmocytoid dendritic cells	Palatine tonsil
$p16^{INK4a}$	Abcam, Cambridge, MA, USA	EPR1473	1:2.000	S2367 (DAKO) pH9	Nonkeratinizing squamous cell carcinoma cells	Palatine tonsil

Table 02. Clinicopathological characteristics of the oral cavity (OSCC) and oropharyngeal (OPSCC) squamous cell carcinoma patients.

Characteristics	OSCC	OPSCC	P
n	109	126	
Age	61.22 ± 12.87	59.06 ± 9.31	0.147*
Sex			
Female	23 (21.10%)	20 (15.87%)	0.3873**
Male	86 (78.90%)	106 (84.13%)	
Tabaco			
Yes	60 (56.60%)	95 (82.60%)	<0.0001**
No	46 (43.40%)	20 (17.40%)	
Alcohol			
Yes	60 (56.60%)	97 (82.90%)	<0.0001**
No	46 (43.40%)	20 (17.10%)	
T-Classification			
T1	12 (13.33%)	15 (15%)	0.2931**
T2	11 (12.22%)	15 (15%)	
T3	38 (42.22%)	50 (50%)	
T4	29 (32.22%)	20 (20%)	
N-Classification			
N0	32 (36.36%)	23 (23.46%)	0.0044** (only for N1 and N2)
N1	26 (29.54%)	16 (16.33%)	
N2	25 (28.40%)	46 (46.94%)	
N3	5 (5.68%)	13 (13.26%)	
Staging			
I	11 (12.5%)	5 (4.90%)	0.0002*** (only for II and IVb)
II	17 (19.31%)	5 (4.90%)	
III	10 (11.36%)	13 (12.74%)	
IVa	44 (50.00%)	52 (50.98%)	
IVb	6 (6.81%)	24 (23.52%)	
IVc	0 (0.00%)	3 (2.94%)	
Radiotherapy			
Yes	81 (75.70%)	96 (81.35%)	0.3837**
No	26 (24.30%)	22 (18.65%)	
Chemotherapy			
Yes	61 (58.65%)	72 (62.60%	0.6456**
No	43 (41.35%)	43 (37.40%)	
Recurrence			
Yes	38 (40.86%)	29 (33.72%)	0.4057**
No	55 (59.14%)	57 (66.28%)	
Metastasis			
Yes	23 (25%)	37 (41.57%)	0.0271**
No	69 (75%)	52 (58.43%)	
HPV			
Negative	97 (89.00%)	94 (75.00%)	0.008**
Positive	12 (11.00%)	32 (25.00%)	

Legends: * Student t test; ** Chi-square test, *** G Test.

Table 3. Clinicopathological features in correlation with human papillomavirus profile and immunohistochemical findings in oral cavity and oropharyngeal squamous cell carcinoma patients.

OSCC (n=109)	Gender (n)	Mean age (y)	Location (n)	Histopathologic grading (n)	$p16^{INK4a}$ positive
HPV-positive n= 12 cases (11%)	M (12)/ F (0)	47	Tongue (5); FOM (4); retromolar area (2); gingiva (1)	WD (7); MD (5)	11/12 (92%)
HRHPV only n=7 cases	M (7)/ Fe (0)	53	Tongue (3); retromolar area (2); gingiva (1); FOM (1)	WD (4); MD (3)	7/7 (100%)
LRHPV only n=3 cases	M (3) F (0)	68	Tongue (1); FOM (2)	WD (2); MD (1)	3/3 (100%)
HRHPV/LRHPV n=2 cases	M (2)/ F (0)	66	Tongue (1); FOM (1)	WD (1); MD (1)	1/2 (50%)
HPV-negative n=97 cases (89%)	M (74)/ F (23)	61	Tongue (19); FOM (38); oral mucosa, NOS (15); retromolar area (9); hard palate (7); alveolar ridge (4); gingiva (3); vestibular fornix (2)	WD (50); MD (44); PD (2)	20/97 (20%)
OPSCC (n=126)	Gender (n)	Mean age (y)	Location (n)	Histopathologic grading (n)	$p16^{INK4a}$ positive
HPV-positive n= 32 cases (25%)	M (25)/ F (7)	60	Palatine tonsil (13); oropharynx NOS (7); BOT (6); uvula (1); soft palate (1); tonsillar pillar (3)	NK (15); H (11); K (6)	29/32 (90%)
WS-HPV only n=4 cases	M (4)/ F (0)	61	Palatine tonsil (2); BOT (1); oropharynx NOS (1)	K (3); NK (1)	4/4 (100%)
HRHPV only n=19 cases	M (15)/ F(4)	57	Palatine tonsil (8); oropharynx NOS (5); BOT (3); uvula (1); tonsillar pillar (1)	NK (11); H (6); K (2)	17/19 (89%)
LRHPV only n=6 cases	M (4)/ F (2)	62	Palatine tonsil (2); tonsillar pillar (2); BOT(1); soft palate (1)	H (5); NK (1)	5/6 (83%)
HRHPV/LRHPV n=3 cases	M (2)/ F (1)	67	Palatine tonsil (1); BOT (1); oropharynx NOS (1)	NK (2); K (1)	3/3 (100%)
HPV-negative n=94 cases (75%)	M (83)/ F (11)	59	Palatine tonsil (21); BOT (20); oropharynx NOS (18); uvula (1); soft palate (14); uvula (7); tonsillar pillar (6)	K (91); NK (2); H (1)	58/94 (62%)

WS HPV, Wide-spectrum human papillomavirus; HRHPV, High-risk human papillomavirus; LRHPV, Low-risk human papillomavirus; HRHPV/LRHPV, high-risk and low-risk human papillomavirus (co-infection);
FOM, floor of the mouth; BOT, base of the tongue; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; K, keratinizing; H, hybrid; NK, nonkeratinizing.
M, Male; F, Female; Y, years
All HRHPV, LRHPV and HRHPV/LRHPV cases were also positive for wide-spectrum (WS)-HPV probe.

Histopathological grading for all OSCC cases: WD (53%), MD (45%), PD (2%).
Histopathological grading for all OPSCC cases: K (77%), NK (13%), H (10%)
HPV-positive patients, mean age, OSCC vs OPSCC (p=0.147)

Table 04. Density of dendritic cell subsets in oral cavity (OSCC) and oropharyngeal (OPSCC) squamous cell carcinoma patients.

Dendritic cell subtype	OSCC (MSC ± DM)	OPSCC (MSC ± DM)	P-value
S100	3.54 ± 3.50	6.97 ± 5.01	<0.0001
CD1a	3.13 ± 3.82	4.23 ± 3.62	0.040
CD207	2.06 ± 2.34	3.71± 3.44	<0.0001
FXIIIa	2.28 ± 3.17	1.60 ± 2.57	0.071
CD209	3.87 ± 5.75	1.63 ±2.62	<0.0001
CD83	0.87 ± 1.35	1.12 ± 1.19	0.123
CD208	0.13 ± 0.28	0.23 ± 0.47	0.037
CD123	1.54 ± 2.13	1.37 ± 1.47	0.496
CD303	0.12 ± 0.32	0.14 ± 0.26	0.592

Legend. MSC: measure of central tendency (mean); DM: dispersion measure (standard deviation).

Table 05. Density of dendritic cell subsets, in correlation with human papillomavirus (HPV) status, in oral cavity squamous cell carcinoma patients.

Dendritic cell subtype	HPV-positive group (MSC ± DM)	HPV-negative group (MSC ± DM)	P-value
S100	4.19 ± 3.23	2.75 ± 3.38	0.1648*
CD1a	2.05 ± 2.00	0.8 ± 4.20	0.2300**
CD207	2.05 ± 2.70	0.7 ± 2.2	0.2319**
FXIIIa	0.95 ± 4.30	0.00 ± 4.20	0.3071**
CD209	1.10 ± 5.70	0.00 ± 8.20	0.9691**
CD83	1.15 ± 1.01	0.82 ± 1.37	0.4323*
CD208	0.00 ± 0.62	0.00 ± 0.00	0.2357**
CD123	0.92 ± 0.75	0.57 ± 0.90	0.2154*
CD303	0.00 ± 0.67	0.00 ± 0.00	0.1896**

Legend. MSC: measure of central tendency (mean or median); DM: dispersion measure (standard deviation or interquartile deviation); *: Student's T test; **: Mann-Whitney test.

Table 06. Density of dendritic cell subsets, in correlation with human papillomavirus (HPV) status, in oropharyngeal squamous cell carcinoma patients.

Dendritic cell subtype	HPV-positive group (MSC ± DM)	HPV-negative group (MSC ± DM)	P-value
S100	6.78 ± 4.21	5.63 ± 5.55	0.226*
CD1a	5.62 ± 4.68	3.59 ± 3.31	0.020*
CD207	4.80 ± 4.39	2.74 ± 3.24	0.018*
FXIIIa	1.88 ± 3.00	1.64 ± 2.73	0.680*
CD209	0.00 ± 2.72	0.00 ± 3.27	0.890**
CD83	1.58 ± 1.52	0.97 ± 1.02	0.740*
CD208	0.10 ± 0.67	0.00 ± 0.17	0.078**
CD123	1.30 ± 1.95	0.95 ± 2.17	0.257**
CD303	0.00 ± 0.42	0.00 ± 0.27	0.221**

Legend. MSC: measure of central tendency (mean or median); DM: dispersion measure (standard deviation or interquartile deviation); *: Student's T test; **: Mann-Whitney test.

Table 07. Density of dendritic cell subsets in human papillomavirus (HPV)-associated oral cavity (OSCC) and oropharyngeal (OPSCC) squamous cell carcinoma patients.

Dendritic cell subtypes	HPV-associated OSCC (MSC ± DM)	HPV-associated OPSCC (MSC ± DM)	P-value
S100	4.19 ± 3.23	6.78 ± 4.21	0.061*
CD1a	2.05 ± 2.00	5.35 ± 5.30	0.077**
CD207	2.29 ± 2.09	4.80 ± 3.39	0.014*
FXIIIa	0.95 ± 4.3	0.05 ± 2.67	0.235**
CD209	1.10 ± 5.70	0.00 ± 2.72	0.240**
CD83	1.15 ± 1.10	1.58 ± 1.52	0.376*
CD208	0.00 ± 0.62	0.10 ± 0.67	0.853**
CD123	0.91 ± 0.75	1.62 ± 1.52	0.046*
CD303	0.00 ± 0.67	0.00 ± 0.42	0.843**

Legend. MSC: measure of central tendency (mean or median); DM: dispersion measure (standard deviation or interquartile deviation); *: T-student's test; **: Mann-Whitney test.

3.2 Publicação 2

LOW-RISK AND HIGH-RISK HUMAN PAPILLOMAVIRUS PROFILE AND CELL PROLIFERATION IN ORAL AND OROPHARYNGEAL SQUAMOUS CELL CARCINOMA IN A BRAZILIAN POPULATION

Heitor Albergoni Silveira[1,2], Luciana Yamamoto Almeida[2], Roman Carlos[3], Evânio Vilela Silva[2], Tulio Morandin Ferrisse[2], Andressa Duarte[3], Suziene Caroline Silva Cardoso[3], Xiomara Beatriz Jimenez Polanco[1], Andreia Bufalino[2], Alfredo Ribeiro-Silva[3], Jorge Esquiche León[1]

[1] Oral Pathology, Department of Stomatology, Public Oral Health, and Forensic Dentistry, Ribeirão Preto Dental School (FORP/USP), University of São Paulo, Ribeirão Preto, SP, Brazil.

[2] Oral Medicine, Department of Diagnosis and Surgery, Araraquara Dental School, São Paulo State University (UNESP), Araraquara, São Paulo, Brazil.

[3] Centro Clínico de Cabeza y Cuello, Guatemala City, Guatemala.

[4] Department of Pathology and Forensic Medicine, Ribeirão Preto Medical School (FMRP/USP), University of São Paulo, Ribeirão Preto, SP, Brazil.

Key-Words: HPV; head and neck squamous cell carcinoma; oropharyngeal; oral cavity; low-risk; high-risk; immunohistochemistry; in situ hybridization.

Funding:

Heitor Albergoni Silveira (2018/12734-2), Luciana Yamamoto Almeida (2016/02713-2) and Jorge Esquiche León (2016/11419-0) have received research Grants from State of São Paulo Research Foundation (FAPESP).

***Corresponding author:** Prof. Dr. Jorge Esquiche León, DDS, MSc, PhD; Oral Pathology, Department of Stomatology, Public Oral Health and Forensic Dentistry, School of Dentistry of Ribeirão Preto, University of São Paulo (FORP/USP), Ribeirão Preto, 14040-904, SP, Brazil. E-mail jleon@forp.usp.br. Tel +55 016 33154063 Fax +55 016 33154794.

---

[*] O artigo segue as normas para publicação no periódico Head & Neck (ISSN: 1097-0347) para o qual pretende-se submeter.

NOTES

Compliance with Ethical Standards

Ethical Approval

This study was reviewed and approved by the Research Ethics Committee of the Ribeirão Preto Medical School, University of São Paulo (Protocol number: 99417018.4.0000.5440) and all procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflicts of interest statement: The authors have no conflict of interest in the present manuscript.

ABSTRACT

BACKGROUND: The interactions between high-risk (HRHPV) and low-risk (LRHPV) human papillomavirus (HPV), as well as its frequency and prognostic impact, in oropharyngeal (OPSCC) and oral (OSCC) squamous cell carcinoma are unknown.

METHODS: Through in situ hybridization (using HPV probes) and immunohistochemistry (using $p16^{INK4a}$, Cyclin D1 and Ki-67 markers), 126 OPSCCs and 109 OSCCs were assessed.

RESULTS: OPSCC (25%) than OSCC (11%) showed significant HPV association. In OPSCC and OSCC, 19 and 7 were HRHPV only, 6 and 3 LRHPV only, and 3 and 2 HRHPV/LRHPV, respectively. HPV-positive than HPV-negative tumors showed significantly higher proliferative index. HRHPV- and LRHPV-associated OSCC showed higher overall survival rate, followed by HRHPV- and LRHPV-associated OPSCC and HPV-unassociated tumors, and lastly by HRHPV/LRHPV-associated cases.

CONCLUSION: LRHPV- and HRHPV/LRHPV-associated cases can be detected in OSCC and OPSCC, with apparent impact on survival. Further studies are necessary to better understand the clinicopathological behavior of these tumor subgroups.

Introduction

The head and neck squamous cell carcinoma (HNSCC) is the eighth most common malignancy worldwide, with 834.000 new cases diagnosed in 2018. About 40%, 25% and 15% of head and neck cancers occur in the oral cavity, larynx, and pharynx, respectively.[1] Squamous cell carcinoma (SCC) is the most common malignancy (95%) in the oral cavity (OSCC), often affecting elderly patients in association with alcohol and tobacco consumption.[2,3] HPV-associated OSCC occurs between 10% to 25% of the cases, being relatively homogeneous across studies, with HPV16 being the most common subtype detected.[1,4,5] Differently, the oropharyngeal squamous cell carcinoma (OPSCC) shows overall a high frequency of HPV association (up to 70% to 80%), with HPV16 being the most prevalent genotype.[6-9] Initial studies have reported the HPV-associated OPSCC as often in nondrinker, nonsmoker young patients, usually with a better survival prognosis when compared with HPV-unassociated OPSCC. However, further studies have showed that OPSCC shows variable clinicopathological features, including HPV prevalence, according to the assessed geographical region.[6,8,9,10-16]

Noteworthy, different from Central and South America (14.9%), North America (54.7%), Asia (45%), Oceania (42.1%) and Europe (36.2%) show high prevalence of HPV-associated OPSCC, being the HPV16 subtype often detected.[11] Data from Brazil show that HPV-associated OPSCC vary between 5.6% to 25.6%,[6,7,10] being similar to Cuba (15.4%), Thailand (14.5%) and Poland (10.7%).[7,10,11,17-21] In contrast, Sweden (85%), New Zeland (78%), Canada (77.9%), Norway (77%), USA (65.2%), and Denmark (62%) show a high prevalence of HPV-associated OPSCC,[8,10-15] whereas Germany (34.4%) shows intermediate rate.[22] In these studies, the high-risk HPV (HRHPV; HPV16 and HPV18 subtypes) was commonly assessed; however, the low-risk HPV (LRHPV) profile, as well as its prognostic impact and possible interactions with HRHPV[5,23] are poorly understood.

In this concern, being unknown for OSCC and OPSCC, some studies assessing cervical cancer and premalignant lesions have shown that HPV co-infection can be associated with a lower rate of developing cervical cancer, probably due to antagonistic role between HRHPV and LRHPV genotypes,[24-27] while others show that HPV co-infection increase cervical cancer risk,[28-30] as well as highlight its association with low response and survival rate of patients under radiotherapy.[31] Moreover, these latter emphasize that Since cervical cancer is often associated with

multiple HPV genotype infection, the co-infection is considered a risk factor, which probably drives carcinogenesis.[32] Accordingly, the identification of cancer-associated HRHPV and LRHPV genotypes is crucial, especially from a preventive standpoint, as HPV vaccine appears to be very effective.[24-32]

Such as above commented, and considering that the LRHPV profile in OSCC and OPSCC needs to be better clarified, which could have a prognostic impact and/or identify a clinicopathological profile, the aim of the current study was to assess the LRHPV and HRHPV profile, in close correlation com $p16^{INK4a}$ expression and cell proliferation index, in a large series of OSCC and OPSCC cases in a Brazilian population.

Material and Methods

Patients and samples

Formalin-fixed, paraffin-embedded tissue blocks from surgical specimens of 126 OPSCCs and 109 OSCCs were obtained from the Laboratory of Anatomical Pathology, Ribeirão Preto Medical School, University of São Paulo (FMRP/USP). A retrospective study was conducted, the clinical data for all cases were obtained from medical records, including the age, gender, ethnicity, clinical stage, TNM, smoking and alcohol consumption status, follow-up duration, outcome and survival status.

This study was approved by the Human Research Ethics Committee from Ribeirão Preto Medical School, University of São Paulo (FMRP/USP) CAAE: #99417018.4.0000.5440.

Histopathological evaluation

The histopathological classification on H&E slides of all cases was established according to the criteria proposed by World Health Organization (WHO, 2017) of head and neck tumors.[33]

The OPSCC cases were histologically classified as keratinizing (K) or nonkeratinizing (NK). The cases presenting focal areas of squamous maturation/keratinization (>10%) were classified as hybrid (H).[21,27] The OSCC cases were classified as well (WD), moderately (MD) and poorly (PD) differentiated.[33]

Construction of Tissue Microarray (TMA)

After morphological review of all the cases, a more representative area of the tumor was selected and later marked. The slides were placed on the original paraffin tissue block to determine the corresponding area to be used in the TMA construction.

The TMA blocks were constructed using a manual tissue matrix (Sakura Co., Tokyo, Japan), each containing cylindrical cores (2.0-mm diameter each).[334,35] Except $p16^{INK4a}$, all antibodies () and probes were assessed on TMA slides. A conclusive result was obtained, if there was no difference between duplicate TMAs.

Immunohistochemical (IHC) technique

TMA blocks were cut to a thickness of three-µm, which were placed on slides properly coated with organosilane (Sigma-Aldrich, St Louis, MO, USA). The slides were submitted to the IHC technique by using the streptavidin-biotin-peroxidase method (K0690; Universal, Dako LSAB®+ Kit, Peroxidase, Carpinteria, CA, USA) to evaluate individual primary antibodies, according to the manufacturer's protocol. Negative control specimens included replacing the primary antibody with isotype-specific serum. The primary antibodies used in the present study were: $p16^{INK4a}$ (clone EPR14, dilution 1:2000, Abcam, Cambridge, MA, USA), cyclin D1 (clone P2D11F11, dilution 1:100, Leica Biosystems, Newcastle, UK) and Ki-67 (clone SP6, dilution 1:500, Abcam, Cambridge, MA, USA).

In situ hybridization (ISH) for HPV and Epstein-Barr virus (EBV)

To determine HPV or EBV infection, all cases were assessed by ISH. Epstein–Barr encoding region (EBER) ISH, was performed with fluorescein isothiocyanate (FITC)-conjugated EBER1/2 PNA-probes (DAKO, code Y5200). Signal amplification was developed with anti-FITC alkaline phosphatase-conjugated antibody and 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate substrate combined with levamisole (DAKO, code K 5201). In situ HPV DNA detection was performed with biotinylated probes Y1404 Wide Spectrum (WS) including HPV genotypes 6, 11, 16, 18, 31, 33, 35, 39, 45, 51 and 52; Y1411 HPV types 6/11 DNA Probe Mix; and Y1443 GenPoint HPV, biotinylated DNA Probe targeting sequences of "high-risk" HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Signal amplification was developed with K620 (GenPoint, Dako, Carpinteria, CA, USA).

Scanning of the TMA slides

The slides were scanned using the programs Pannoramic Midi Digital Slide Scanner (3DHISTECH Ltd., Budapest, Hungary), Pannoramic MIDI Scanners (3DHISTECH Ltd., Budapest, Hungary).

Scoring

Two expert pathologists (A.R.S and J.E.L) evaluated all TMA slides, in cases of disagreement, a consensus classification was determined. For $p16^{INK4a}$ antibody, nuclear and cytoplasmic positive immunostaining patterns, was scored as positive (>70%) or negative/equivocal (<70%).[36] For Ki-67 and Cyclin D1 antibodies, the slides were first evaluated at ×100 magnification, after this, five areas with higher immunoexpression were selected and registered in a microscopic field at ×400 magnification, through an optical microscope (Leica DM500) adapted to a high-resolution camera (Leica IC50). All positive and negative cells in the captured images were quantified, and a percentage per cylinder was determined, using Image J software (National Institutes of Health, Bethesda, MD). For EBER ISH, samples were scored as positive reaction if a purple color in the tumor nuclei was observed. For HPV ISH, the presence of dark/brown punctate (integrated) or diffuse (episomal) nuclear signals in the tumor nuclei were interpreted as positive reaction.[37]

Statistical analysis

Statistical analysis was performed using the IBM SPSS 20.0 software. The Kolmogorov-Smirnov test to check the data normality and the Levene test to check the data homoscedasticity were used. Moreover, the Student´s T test, G test and the chi-square test were applied when indicated. Kaplan-Meir test in association with log-rank, as well as univariate and multivariate Cox regression, for survival analysis were performed.

RESULTS

The clinicopathological features are shown in the Table 1. The Tables 2 and 3 show the survival characteristics. The Table 4 shows the specific anatomical location, histopathological grading (HG), HPV profile and IHC findings. The Table 5 shows the clinicopathological features of the patients with LRHPV and HRHPV/LRHPV profile.

Without differences regarding age (mean, 60 years) and gender (male: female ratio 4-5:1), alcohol and tobacco consumption were significantly more frequent in OPSCC than OSCC. Considering the TNM staging, T stage was similar, whereas N0-N1 and N2-N3 was more frequent in OSCC and OPSCC, respectively. Metastasis was significantly higher in OPSCC than OSCC ($p<0.05$), whereas recurrence was more often observed in OSCC than OPSCC ($p>0.05$). HPV association and $p16^{INK4a}$ positivity were significantly higher in OPSCC than OSCC ($p<0.05$). There were no statistical differences when comparing the HPV subgroups. Considering the cell

proliferation markers, the Ki-67 ($p>0.05$) and Cyclin D1 ($p<0.05$) expression was higher in OPSCC than OSCC (Table 1). By considering the survival characteristics of OSCC and OPSCC patients, in general, there were no statistical differences, mainly including the HPV status. However, only in OPSCC patients, radiotherapy was significantly correlated with a better survival ($p=0.018$), followed by chemotherapy ($p=0.087$), as well as alcohol ($p=0.049$) and tobacco ($p=0.012$) consumption (Tables 2 and 3).

In the OSCC group, 12 (11%) cases (all male) were HPV positive. These patients (mean age, 47 years) were younger than HPV-negative patients (mean age, 61 years) ($p=0.0575$). The most common locations were tongue and floor of the mouth (FOM), exhibiting similar HG. All but one HPV-positive case was $p16^{INKa}$ positive. Notably, $p16^{INKa}$ positivity was also detected in 20% of HPV-negative patients. The Ki-67 ($p<0.05$) and Cyclin D1 ($p<0.001$) labeling index was significantly higher in HPV-positive than HPV-negative patients. Of 12 HPV-positive cases, 7 were HRHPV-positive only, 3 LRHPV-positive only and 2 HRHPV/LRHPV-positive (co-infection). The HRHPV positive group (mean age, 53 years) was younger than LRHPV and HRHPV/LRHPV groups (mean age, 66 years) ($p=0.167$). In contrast, these latter showed a higher Ki-67 labeling index than the HRHPV patients. The Cyclin D1 labeling index was variable, however higher in the LRHPV group (Table 4)

In the OPSCC group, 32 (25%) cases (male:female ratio 3.6:1) were HPV-positive. Different from OSCC, the mean age of HPV-positive and HPV-negative patients was similar. The most common locations were palatine tonsil, base of the tongue (BOT) and oropharynx NOS. The HG between the HPV-positive (NK, 15; H, 11; K, 6) and HPV-negative group (K, 91; NK, 2; H, 1) was opposite. $p16^{INKa}$ expression was observed in all but three HPV-positive cases, as well as in 62% of HPV-negative cases. Similar with OSCC, the Ki-67 and Cyclin D1 labeling index was significantly higher in HPV-positive than HPV-negative patients. Of 32 HPV-positive cases, 19 were HRHPV positive, 6 LRHPV positive, 3 HRHPV/LRHPV positive (co-infection) and 4 were WS-HPV positive only. The HRHPV (mean age, 57 years) were younger than LRHPV and HRHPV/LRHPV (mean age, 64 years) and WS-HPV (mean age, 61 years) groups ($p=0.087$). In contrast, the HRHPV group showed a lower Ki-67 labeling index than all other HPV-positive groups. The Cyclin D1 labeling index was variable, but higher in the LRHPV group (Table 4).

The Table 5 shows the main clinicopathological features of HRHPV/LRHPV and LRHPV cases. Different from HRHPV/LRHPV, it is evident the alcohol and tobacco consumption in LRHPV patients. Considering the HG, the WDSCC was predominant in the oral cavity, whereas the NKSCC for HRHPV/LRHPV and HSCC for LRHPV in the oropharynx. Overall without statistically significant differences, noteworthy, a low overall survival rate was observed in HRHPV/LRHPV-associated OSCC and OPSCC, when compared to other groups. Moreover, a similar overall survival rate comparing HRHPV and LRHPV cases within each OSCC and OPSCC was observed. The overall survival rate in HPV-unassociated OSCC and OPSCC patients was similar, and comparable with HRHPV- and LRHPV-associated OPSCC patients.

DISCUSSION

In the current study, OPSCC (25%) than OSCC (11%) showed significant HPV association. Considering only specific oncogenic genotype, OPSCC (15%) than OSCC (6%) showed significant association with HRHPV. Moreover, we show for the first time, that LRHPV- and HRHPV/LRHPV-associated cases can be detected when assessing OSCC and OPSCC, with apparent impact on survival. Noteworthy, we have observed that HRHPV- and LRHPV-associated OSCC showed a higher overall survival rate, followed by HRHPV- and LRHPV-associated OPSCC and HPV-unassociated tumors, and lastly by HRHPV/LRHPV-associated cases in both neoplasms.

While the HPV prevalence in OSCC appears to be uniform across most studies worldwide,[1,4,20] for OPSCC is variable, with clinicopathological differences, depending on the geographical region assessed. Thus, different from initial studies, HPV-associated OPSCC can also be prevalent in smoker, drinker older patients, with HPV association varying between 10% to 80%. In most studies, the HPV16 is more prevalent when compared with other HRHPV genotype.[7-22] Notably, the LRHPV profile, as well as its prognostic impact and possible interactions with HRHPV, are poorly understood in OSCC and OPSCC. In this context, there are only two studies,[5,23] which superficially assess LRHPV genotypes in OSCC.

The HPV co-infection with multiple genotypes probably promotes the progression of cervical cancer and premalignant lesions.[30] Co-infection among HPV

genotypes is common in males and females, but their clinical significance still remains controversial, and the epidemiology of HPV genotype combinations is unknown. In fact, while some studies have shown that HPV co-infection (among HRHPV genotypes, followed by HRHPV and LRHPV genotypes) increases cervical cancer risk[28-30] and its association with a low survival rate in cervical cancer patients,[31] other studies have shown that HPV co-infection can be associated with a lower rate of developing cervical cancer, probably due to antagonistic role between HRHPV and LRHPV genotypes.[24-27] Our results show that HPV co-infection, considering the positive expression of both HRHPV and LRHPV probes, in OSCC and OPSCC, is associated with low survival rate, similar com previous findings.[28-31] However, different from studies evaluating samples of cervical cancer and premalignant lesions, in the current study we have used HPV probes by ISH; therefore, the exact typification of the individual HPV genotypes was not possible. Moreover, similar with HPV-associated genital carcinoma, further studies focusing OSCC and OPSCC cases should be carried out to determine the prevalence and prognostic impact of the interaction between HRHPV and LRHPV genotypes.

Another issue that needs to be clarified is the similar overall survival rate observed in the current HRHPV and LRHPV cases, in both neoplasms. These findings are unusual, due to the recognized oncogenic potential of these genotypes and its differences on survival rates [26,30]; however, considering the few cases detected in this series, it encourages future studies to assess its true prognostic impact, including those regions where the prevalence of HPV-associated OPSCC is considered high. Nevertheless, from our results, the high expression of Cyclin D1 observed in LRHPV-associated OSCC and OPSCC cases, when compared with other HPV-associated groups, could indicate interactions of this HPV genotype on the mechanisms of cell proliferation, being possible to partially explain these findings.

Other finding relevant was that HPV-associated OPSCC and HPV-unassociated neoplasms, showed similar overall survival; however, it was lower that HPV-associated OSCC, in the current series. Considering that N2-N3 status and metastasis were more frequently observed in OPSCC and OSCC, which can to explain at least in part these findings, other clinicopathological variables, such as shown in the Tables 1-3, including specific anatomical locations, surgical techniques and adjuvant therapies, should also be considered. Moreover, recent findings in a

Brazilian population have evidenced that the HPV influence on survival of OPSCC patients is not clarified, being necessary other studies on this topic.[10]

By considering the HG, in the OSCC the WDSCC and MDSCC exhibited a similar proportion, with very few PDSCC cases detected. In the OPSCC, the HG was dependent of the HPV status. Thus, in the HPV-associated OPSCC, the NKSCC was predominant, followed by HSCC and KSCC, whereas in the HPV-unassociated OPSCC, the most cases were KSCC. Moreover, almost all these NKSCC and HSCC cases in both neoplasms were positive for $p16^{INK4a}$. However, it is evident the notorious difference when considered the prevalence of NKSCC in OPSCC series. Thus, while in the current series was 13% (17/126), other studies report 55%[38] and 80%[9]. This could explain the low HPV prevalence in our OPSCC series, because strictly defined NKSCC in oropharynx essentially implies positivity for both $p16^{INK4a}$ and HPV.[30]

In summary, beyond the typical HRHPV-associated cases, our results show that LRHPV- and HRHPV/LRHPV-associated cases can be detected in OSCC and OPSCC, with an apparent impact on survival. The pathogenic mechanisms of multiple HPV infection, however, still remain uncertain. Further studies are needed to better understand the clinicopathological behavior and prognostic impact of these tumor subgroups.

Figures:

Figure 1: Histopathological grading of oral cavity squamous cell carcinoma: (A) well differentiated; (B) moderately differentiated and (C) poorly differentiated. Histopathological grading oropharyngeal squamous cell carcinoma: (D) keratinizing (E) nonkeratinizing with maturation (hybrid); (F) nonkeratinizing (hematoxylin and eosin stain, original magnification x200).

Figure 2: I In situ hybridization analysis showing nuclear punctate signal pattern in wide-spectrum (WS) human papillomavirus (HPV) (A); high-risk HPV (B); low-risk HPV (C); Co-infection cases were considered when they were positive for HRHPV and LRHPV (including WSHPV), as illustrated in the figures D-F. Immunopositivity for $p16^{INK4a}$ was considered for cases with > 70% of immunostaining (G). The cell

proliferation markers were Cyclin D1 (H) and Ki-67 (I), which showed high expression in HPV-associated neoplasms.

References

1. Hübbers CU, Akgül B. HPV and cancer of the oral cavity. Virulence. 2015;6:244-8.

2. Han N, Zhang Z, Liu S, et al. Increased tumor-infiltrating plasmacytoid dendritic cells predicts poor prognosis in oral squamous cell carcinoma. Arch Oral Biol. 2017;78:129-134.

3. Panarese I, Aquino G, Ronchi A, et al. Oral and Oropharyngeal squamous cell carcinoma: prognostic and predictive parameters in the etiopathogenetic route. Expert Rev Anticancer Ther. 2019;19:105-119.

4. Syrjänen S, Lodi G, von Bültzingslöwen I, et al. Human papillomaviruses in oral carcinoma and oral potentially malignant disorders: a systematic review. Oral Dis. 2011;17 Suppl 1:58-72.

5. Anaya-Saavedra G, Ramírez-Amador V, Irigoyen-Camacho ME, et al. High association of human papillomavirus infection with oral cancer: a case-control study. Arch Med Res. 2008;39:189-197.

6. Barros-Filho MC, Reis-Rosa LA, Hatakeyama M, et al. Oncogenic drivers in 11q13 associated with prognosis and response to therapy in advanced oropharyngeal carcinomas. Oral Oncol. 2018;83:81-90.

7. Piña AR, Jimenez LS, Mariano FV, et al. Human papillomavirus in tonsillar squamous cell carcinomas from Guatemala and Brazil. Oral Surg Oral Med Oral Pathol Oral Radiol. 2016;121:412-418.

8. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol. 2011;29:4294-301.

9. Thompson LDR, Burchette R, Iganej S, Bhattasali O. Oropharyngeal Squamous Cell Carcinoma in 390 Patients: Analysis of Clinical and Histological Criteria Which Significantly Impact Outcome. Head Neck Pathol. 2019 Nov 18. [Epub of head]

10. De Cicco R, de Melo Menezes R, Nicolau UR, Pinto CAL, Villa LL, Kowalski LP. Impact of human papillomavirus status on survival and recurrence in a

geographic region with a low prevalence of HPV-related cancer: A retrospective cohort study. Head Neck. 2020;42:93-102.

11. Ndiaye C, Mena M, Alemany L, et al. HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. Lancet Oncol. 2014;15:1319-1331.

12. Fossum GH, Lie AK, Jebsen P, Sandlie LE, Mork J. Human papillomavirus in oropharyngeal squamous cell carcinoma in South-Eastern Norway: prevalence, genotype, and survival. Eur Arch Otorhinolaryngol. 2017;274:4003-4010.

13. Carlander AF, Grønhøj Larsen C, Jensen DH, et al. Continuing rise in oropharyngeal cancer in a high HPV prevalence area: A Danish population-based study from 2011 to 2014. Eur J Cancer. 2017;70:75-82.

14. Liederbach E, Kyrillos A, Wang CH, Liu JC, Sturgis EM, Bhayani MK. The national landscape of human papillomavirus-associated oropharynx squamous cell carcinoma. Int J Cancer. 2017;140:504-512.

15. Lu XJD, Liu KYP, Soares RC, Thomson T, Prisman E, Wu J, Poh CF. Potential clinical implications of HPV status and expressions of p53 and cyclin D1 among oropharyngeal cancer patients. J Oral Pathol Med. 2018;47:945-953.

16. Lucas-Roxburgh R, Benschop J, Dunowska M, Perrott M. Prevalence of human papillomaviruses in the mouths of New Zealand women. N Z Med J. 2015;128:45-52.

17. Li W, Thompson CH, Xin D, Cossart YE, et al. Absence of human papillomavirus in tonsillar squamous cell carcinomas from Chinese patients. Am J Pathol. 2003;163:2185-2189.

18. Ribeiro KB, Levi JE, Pawlita M, et al. Low human papillomavirus prevalence in head and neck cancer: results from two large case-control studies in high-incidence regions. Int J Epidemiol. 2011;40:489-502.

19. Baboci L, Holzinger D, Boscolo-Rizzo P, et al. Low prevalence of HPV-driven head and neck squamous cell carcinoma in North-East Italy. Papillomavirus Res. 2016;2:133-140.

20. Matos LL, Miranda GA, Cernea CR. Prevalence of oral and oropharyngeal human papillomavirus infection in Brazilian population studies: a systematic review. Braz J Otorhinolaryngol. 2015;81:554-67.

21. Nopmaneepaisarn T, Tangjaturonrasme N, Rawangban W, Vinayanuwattikun C, Keelawat S, Bychkov A. Low prevalence of p16-positive HPV-related head-neck cancers in Thailand: tertiary referral center experience. BMC Cancer. 2019;19:1050

22. Hauck F, Oliveira-Silva M, Dreyer JH, et al. Prevalence of HPV infection in head and neck carcinomas shows geographical variability: a comparative study from Brazil and Germany. Virchows Arch. 2015;466:685-693.

23. Duray A, Descamps G, Decaestecker C, et al. Human papillomavirus DNA strongly correlates with a poorer prognosis in oral cavity carcinoma. Laryngoscope. 2012;122:1558-1565.

24. Arnheim Dahlstrom L, Andersson K, et al. Prospective seroepidemiologic study of human papillomavirus and other risk factors in cervical cancer. Cancer Epidemiol Biomarkers Prev. 2011;20:2541–2550.

25. Sundström K, Ploner A, Arnheim-Dahlström L, et al. Interactions Between High- and Low-Risk HPV Types Reduce the Risk of Squamous Cervical Cancer. J Natl Cancer Inst. 2015;107:pii: djv185.

26. Luostarinen T, af Geijersstam V, Bjørge T, et al. No excess risk of cervical carcinoma among women seropositive for both HPV16 and HPV6/11. Int J Cancer. 1999;80:818-822.

27. Wentzensen N, Nason M, Schiffman M, Dodd L, Hunt WC, Wheeler CM; New Mexico HPV Pap Registry Steering Committee. No evidence for synergy between human papillomavirus genotypes for the risk of high-grade squamous intraepithelial lesions in a large population-based study. J Infect Dis. 2014;209:855-864.

28. Dahlström LA, Ylitalo N, Sundström K, et al. Prospective study of human papillomavirus and risk of cervical adenocarcinoma. Int J Cancer. 2010;127:1923-1930.

29. Trottier H, Mahmud S, Costa MC, et al. Human papillomavirus infections with multiple types and risk of cervical neoplasia. Cancer Epidemiol Biomarkers Prev. 2006;15:1274-1280.

30. Gallegos-Bolaños J, Rivera-Domínguez JA, Presno-Bernal JM, Cervantes-Villagrana RD. High prevalence of co-infection between human papillomavirus (HPV) 51 and 52 in Mexican population. BMC Cancer. 2017;17:531.

31. Bachtiary B, Obermair A, Dreier B, et al. Impact of multiple HPV infection on response to treatment and survival in patients receiving radical radiotherapy for cervical cancer. Int J Cancer. 2002;102:237-243.

32. Martora F, Della Pepa ME, Grimaldi E, et al. Seven years prevalence and distribution of high and low risk HPV genotypes in women living in the metropolitan area of Naples. Cancer Epidemiol. 2019;63:101625.

33. El Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ. Classification of Head and Neck Tumours. 4th ed. Lyon, France: IARC Press; 2017.

34. Radhakrishnan R, Solomon M, Satyamoorthy K, Martin LE, Lingen MW. Tissue microarray - a high-throughput molecular analysis in head and neck cancer. J Oral Pathol Med. 2008;37:166-176.

35. Neves-Silva R, Almeida LY, Silveira HA, et al. SMARCB1 (INI-1) and NUT immunoexpression in a large series of head and neck carcinomas in a Brazilian reference center. Head Neck. 2019 Nov 15.

36. Božinović K, Sabol I, Rakušić Z, et al. HPV-driven oropharyngeal squamous cell cancer in Croatia - Demography and survival. PLoS One. 2019;14:e0211577.

37. Wang XI, Thomas J, Zhang S. Changing trends in human papillomavirus-associated head and neck squamous cell carcinoma. Ann Diagn Pathol. 2012;16:7-12.

38. Gondim DD, Haynes W, Wang X, Chernock RD, El-Mofty SK, Lewis JS Jr. Histologic Typing in Oropharyngeal Squamous Cell Carcinoma: A 4-Year Prospective Practice Study With p16 and High-Risk HPV mRNA Testing Correlation. Am J Surg Pathol. 2016;40:1117-1124.

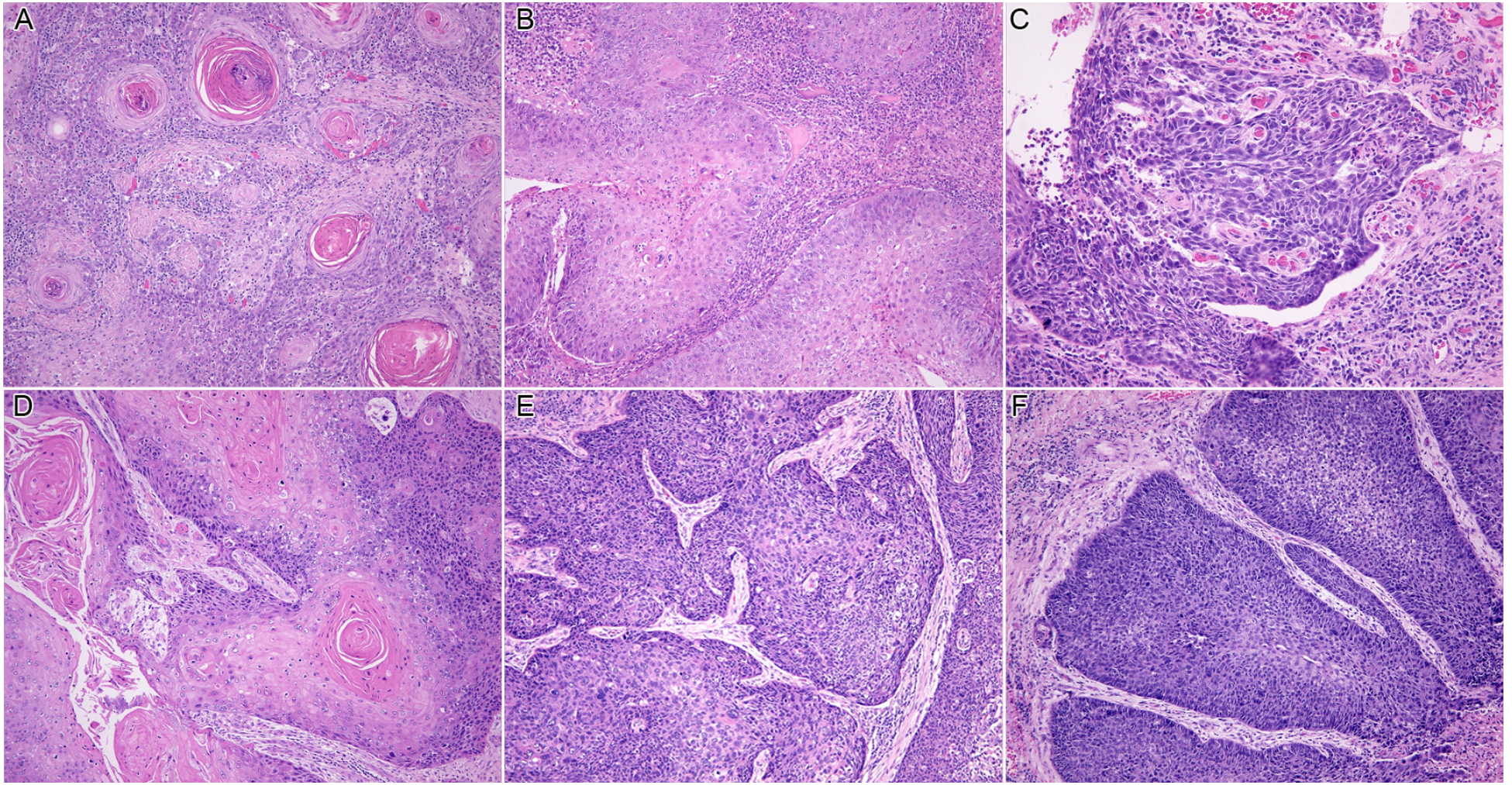

Figure 1: Histopathological grading of oral cavity squamous cell carcinoma: (A) well differentiated; (B) moderately differentiated and (C) poorly differentiated. Histopathological grading oropharyngeal squamous cell carcinoma: (D) keratinizing (E) nonkeratinizing with maturation (hybrid); (F) nonkeratinizing (hematoxylin and eosin stain, original magnification x200).

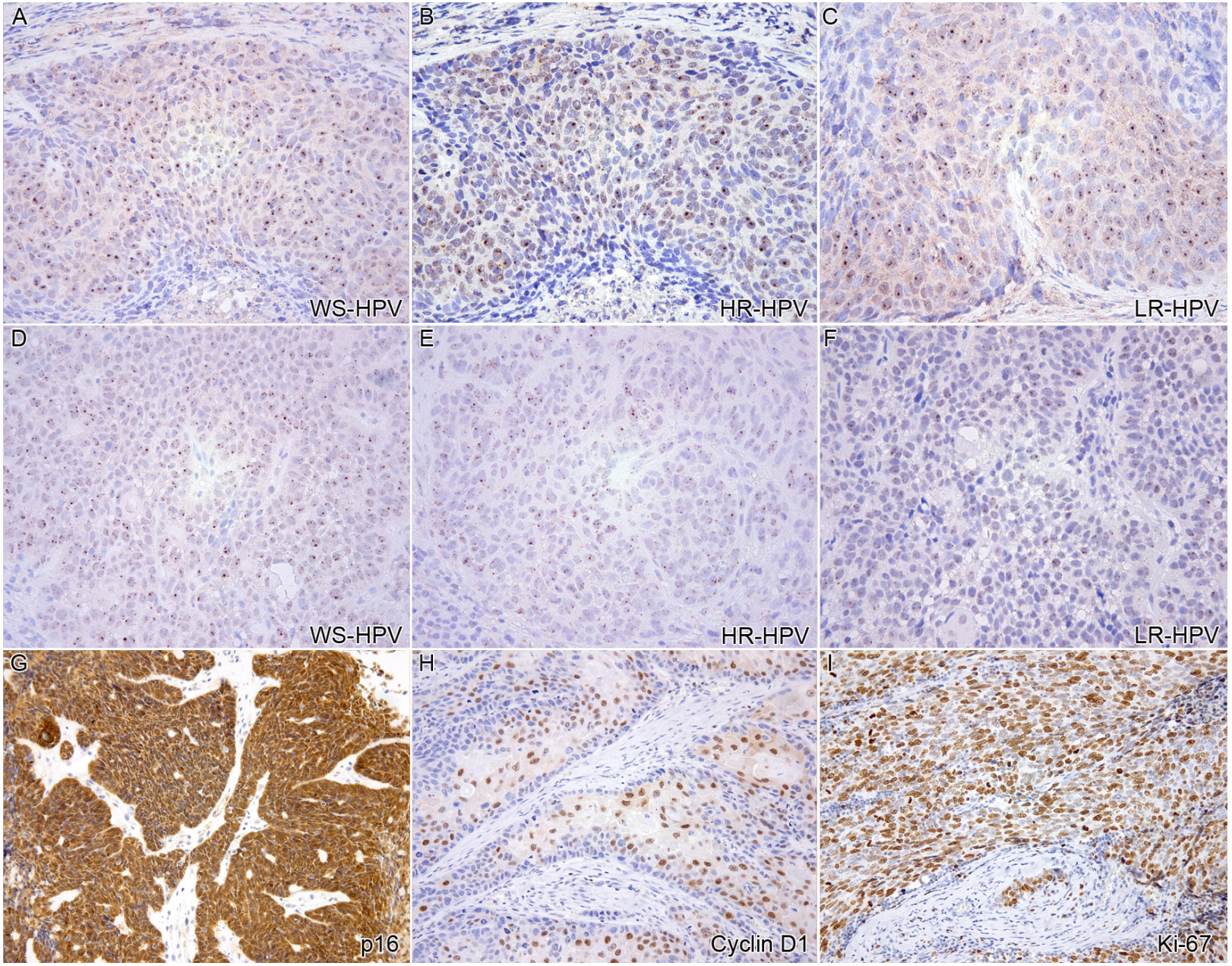
A
WS-HPV
B
HR-HPV
C
LR-HPV
D
WS-HPV
E
HR-HPV
F
LR-HPV
G
p16
H
Cyclin D1
I
Ki-67

Figure 2: In situ hybridization analysis showing nuclear punctate signal pattern in wide-spectrum (WS) human papillomavirus (HPV) (A); high-risk HPV (B); low-risk HPV (C); Co-infection cases were considered when they were positive for HRHPV and LRHPV (including WSHPV), as illustrated in the figures D-F. Immunopositivity for $p16^{INK4a}$ was considered for cases with > 70% of immunostaining (G). The cell proliferation markers were Cyclin D1 (H) and Ki-67 (I), which showed high expression in HPV-associated neoplasms.

Table 1. Characteristics of the Brazilian population affected with squamous cell carcinoma

Characteristics	*Oropharynx*	*Oral cavity*	*P*
Number of cases	123	109	
Age	59.06 ± 9.31	61.22 ± 12.87	0.147*
Sex			
Female	20 (15.87%)	23 (21.10%)	0.3873**
Male	106 (84.13%)	86 (78.90%)	
Tabaco			
Yes	95 (82.60%)	60 (56.60%)	<0.0001**
No	20 (17.40%)	46 (43.40%)	
Alcohol			
Yes	97 (82.90%)	60 (56.60%)	<0.0001**
No	20 (17.10%)	46 (43.40%)	
T-Classification			
T1	15 (15%)	12 (13.33%)	0.2931**
T2	15 (15%)	11 (12.22%)	
T3	50 (50%)	38 (42.22%)	
T4	20 (20%)	29 (32.22%)	
N-Classification			
N0	23 (23.46%)	32 (36.36%)	0.0044** (only for N1 and N2)
N1	16 (16.33%)	26 (29.54%)	
N2	46 (46.94%)	25 (28.40%)	
N3	13 (13.26%)	5 (5.68%)	
Staging			
I	5 (4.90%)	11 (12.5%)	0.0002*** (only for II and IVb)
II	5 (4.90%)	17 (19.31%)	
III	13 (12.74%)	10 (11.36%)	
IVa	52 (50.98%)	44 (50.00%)	
IVb	24 (23.52%)	6 (6.81%)	
IVc	3 (2.94%)	0 (0.00%)	
Radiotherapy			
Yes	96 (81.35%)	81 (75.70%)	0.3837**
No	22 (18.65%)	26 (24.30%)	
Chemotherapy			
Yes	72 (62.60%	61 (58.65%)	0.6456**
No	43 (37.40%)	43 (41.35%)	
Recurrence			
Yes	29 (33.72%)	38 (40.86%)	0.4057**
No	57 (66.28%)	55 (59.14%)	
Metastasis			
Yes	37 (41.57%)	23 (25%)	0.0271**
No	52 (58.43%)	69 (75%)	
HPV			
Negative	94 (75.00%)	97 (89.00%	0.008**
Positive	32 (25.00%)	12 (11.00%)	

Legends: * Statistic analyses were performed by student's T test; ** Statistics analyses were performed by Chi square test, *** Statistics analyses were performed by G Test.

Table 1. Continued.

Characteristics	*Oropharynx*	*Oral cavity*	*P*
HPV			
HRHPV	19 (67.85%)	7 (58.33%)	
LRHPV	6 (21.42%)	3 (25.00%)	0.9000***
HRHPV/LRHPV	3 (10.71%)	2 (16.66%)	
p16^{INK4a}			
Negative	24 (21.05%)	31 (34.44%)	0.0476**
Positive	90 (78.94%)	59 (65.55%)	
Ki-67			
≤10%	22(18.49%)	26 (26.54%)	0.2757**
>10%	97 (81.51%)	73 (73.46%)	
Cyclin D1			
≤10%	100 (80.00%)	99 (90.82%)	0.0330**
>10%	25 (25.00%)	10 (9.18%)	

Legends: * Statistic analyses were performed by student's T test; ** Statistics analyses were performed by Chi square test, *** Statistics analyses were performed by G Test.

Table 2. Survival characteristics of the oral cavity squamous cell carcinoma patients.

	Kaplan-Meier(Long-rank)		*Cox regression*	
Characteristics	*HR (95% CI)*	*P*	*HR (95% CI)*	*P*
Sex				
Female	1.667(0.970-2.363)	0.678	0.908(0.478-1.725)	0.769
Male	1.844(1.593-2.094)			
Tabaco				
Yes	1.840(1.528-2.152)	0.512	1.127(0.669-1.899)	0.654
No	1.645(1.344-1.947)			
Alcohol				
Yes	1.840(1.528-2.152)	0.388	0.855(0.511-1.432)	0.551
No	1.609(1.306-1912)			
T-Classification				
T1	2.750(0.730-4.770)	0.119	-	-
T2	1.529(1.213-1.845)		0.856(0.342-2.141)	0.739
T3	1.667(1.013-2.320)		0.780(0.426-1.428)	0.421
T4	1.815-1.510-2.119)		0.314(0.720-1.375)	0.124
N-Classification				
N0	1.765(1.322-2.207)	0.646	-	-
N1	1.545(1.061-2.030)		0.769(0.350-1.692)	0.514
N2	1.989(1.541-2.436)		1.276(0.353-4.609)	0.710
N3	1.333(0.680-1.987)		0.889(0.425-1.860)	0.755
Radiotherapy				
Yes	1.798(1.546-2.051)	0.957	1.012(0.536-1.913)	0.970
No	1.750(1.062-2.438)			
Chemotherapy				
Yes	1.782(1.478-2.087)	0.652	1.085(0.649-1.814)	0.755
No	1.833(1.413-2.253)			
Recurrence				
Yes	1.957(1.563-2.357)	0.535	0.890(0.517-1.531)	0.673
No	1.731(1.361-2.100)			
Staging				
I	2.333(0.000-4.947)	0.815	1.703(0.470-6.169)	0.418
II	1.604(0.934-2.274)		1.674(0.377-7.442)	0.498
III	1.600(0.816-2.384)		1.479(0.417-5.239)	0.544
IVa	1.833(1.506-2.159)		1.570(0.297-8.295)	0.595
IVb	1.667(1.013-2.320)		-	-
IVc	-		-	
Metastasis				
Yes	1.647(1.354-1.941)	0.341	1.209(0.679-2.151)	0.519
No	1.913(1.548-2.277)			
HPV				
Positive	2.600(1.271-3.929)	0.095	1.719(0.667-4.432)	0.262
Negative	1.745(1.460-2.030)			
HPV				
HRHPV	2.000(0.868-3.132)	0.333	-	-
LRHPV	5.000(5.000-5.000)			
HRHPV/LRHPV	2.000(2.000-2.000)			
p16^{INK4a}				
Positive	1.797(1.535-2.058)	0.992	0.997(0.441-2.254)	0.994
Negative	1.818(0.648-2.988)			
Ki-67				
≤10%	1.870(1.403-2.336)	0.647	1.092(0.633-1.884)	0.752
>10%	1.761(1.000-1.000)			
Cyclin D1				
>10%	1.795(1.520-2.071)	0.919	0.963 (0.349-2.658)	0.942
≤10%	1.750(0.812-2.688)			
HG				
WD	1.669(1.382-1.957)	0.412	0.790(0.476-1.311)	0.361
MD	1.959(1.558-2.360)		0.765(0.104-5.6.6)	0.792
PD	2.000(2.000-2.000)		-	-

Legends. HR: hazard ratio; CI: confidence interval; HRHPV, High-risk human papillomavirus; LRHPV, Low-risk human papillomavirus; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; HG, histopathological grading

Table 3. Survival characteristics of oropharyngeal squamous cell carcinoma patients.

	Kaplan-Meier(Long-rank)		*Cox regression*	
Characteristics	*HR (95% CI)*	*P*	*HR (95% CI)*	*P*
Sex				
Female	1.714(1.154-2.274)	0.579	1.155(0.525-2.540)	0.720
Male	1.590(1.354-1.827)			
Tabaco				
Yes	1.753(1.507-1.999)	0.012	1.897(0.948-3.797)	0.071
No	1.091(0.913-1.269)			
Alcohol				
Yes	1.791(1.507-2.075)	0.049	1.458(0.839-2.537)	0.181
No	1.300(1.050-1.550)			
T-Classification				
T1	2.000(0.482-3.518)		-	-
T2	1.742(1.357-2.128)	0.774	0.676(0.201-2.275)	0.527
T3	1.633(1.123-2.144)		0.870(0.393-1.926)	0.732
T4	1.671(1.313-2.030)		0.979(0.386-2.482)	0.965
N-Classification				
N0	2.019(1.414-2.625)		-	-
N1	2.250(1.699-2.801)	0.523	1.539(0.573-4.137)	0.393
N2	1.614(1.208-2.020)		1.952(0.586-6.501)	0.276
N3	1.333(0.920-1.747)		1.225(0.417-3.601	0.712
Staging				
I	1.000(1.000-1.000)		-	-
II	2.000(2.000-2.000)		-	-
III	1.692(1.239-2.145)	0.282	0.623(0.132-2.934)	0.550
Iva	1.821(1.433-2.209)		0.410(0.116-1.447)	0.166
IVb	1.622(1.110-2.133)		0.510(0.140-1.854)	0.307
IVc	1.000(1.000-1.000)		-	-
Radiotherapy				
Yes	1.749(1.497-2.000)	0.018	1.715(0.903-3.257)	0.099
No	1.154(0.950-1.358)			
Chemotherapy				
Yes	1.804(1.493-2.116)	0.087	1.358(0.800-2.304)	0.257
No	1.375(1.145-1.605)			
Recurrence				
Yes	1.666(1.324-2.009)	0.361	0.833(0.453-1.531)	0.557
No	1.450(1.072-1.829)			
Metastasis				
Yes	1.593(1.173-2.014)	0.789	1.054(0.574-1.935)	0.865
No	1.565(1.267-1.863)			
HPV				
Positive	1.613(1.293-1.933)	0.825	1.042(0.584-1.860)	0.889
Negative	1.559(1.324-1.794)			
HPV			0.941(0.259-3.421)	0.927
HRHPV	1.611(1.177-2.044)	0.721	1.988(0.234-16.896)	0.983
LRHPV	1.667(1.013-2.320)			
HRHPV/LRHPV	1.000(1.000-1.000)		-	-
p16^{INK4a}				
Positive	1.526(1.343-1.708)	0.690	1.127(0.510-2.491)	0.768
Negative	1.625(0.890-2.360)			
Ki-67				
≤10%	1.364(1.017-1.711)	0.280	0.813(0.449-1.472)	0.494
>10%	1.590(1.368-1.812)			
Cyclin D1				
≤10%	1.593(1.369-1.816)	0.939	1.016(0.549-1.882)	0.960
>10%	1.673(1.106-2.239)			
HG				
K	1.624(1.397-1.851)		1.491(0.628-3.539)	0.365
H	1.167(0.840-1.493)	0.741	-	-
NK	1.719(1.009-2.429)		0.979(0.504-1.903)	0.951

Legends. HR: hazard ratio; CI: confidence interval; HRHPV, High-risk human papillomavirus; LRHPV, Low-risk human papillomavirus; K, keratinizing; H, hybrid; NK, non-keratinizing; HG, histopathological grading

Table 4. Clinicopathological features in correlation with human papillomavirus profile and immunohistochemical findings in oral cavity and oropharyngeal squamous cell carcinoma patients.

OSCC (n=109)	Gender (n)	Mean age (y)	Location (n)	Histopathologic grading (n)	$p16^{INK4a}$ positive	Proliferative index	
						Ki-67 (%)	Cyclin D1 (%)
HPV-positive n= 12 cases (11%)	M (12)/ F (0)	47	Tongue (5); FOM (4); retromolar area (2); gingiva (1)	WD (7); MD (5)	11/12 (92%)	46	13 (4/12 cases negative [33%])
HRHPV only n=7 cases	M (7)/ F (0)	53	Tongue (3); retromolar area (2); gingiva (1); FOM (1)	WD (4); MD (3)	7/7 (100%)	27	12 (2/7 cases negative)
LRHPV only n=3 cases	M (3)/ F (0)	68	Tongue (1); FOM (2)	WD (2); MD (1)	3/3 (100%)	44	24 (1/3 case negative)
HRHPV/LRHPV n=2 cases	M (2)/ F (0)	66	Tongue (1); FOM (1)	WD (1); MD (1)	1/2 (50%)	67	4 (1/2 case negative)
HPV-negative n=97 cases (89%)	M (74)/ F (23)	61	Tongue (19); FOM (38); oral mucosa, NOS (15); retromolar area (9); hard palate (7); alveolar ridge (4); gingiva (3); vestibular fornix (2)	WD (50); MD (44); PD (2)	20/97 (20%)	30	3 (58/97 cases [59%])
OPSCC (n=126)	Gender (n)	Mean age (y)	Location (n)	Histopathologic grading (n)	$p16^{INK4a}$ positive	Proliferative index	
						Ki-67 (%)	Cyclin D1 (%)
HPV-positive n= 32 cases (25%)	M (25)// F (7)	60	Palatine tonsil (13); oropharynx NOS (7); BOT (6); uvula (1); soft palate (1); tonsillar pillar (3)	NK (15); H (11); K (6)	29/32 (90%)	56	10 (2/32 cases negative [6%])
WS-HPV only n=4 cases	M (4)/ F (0)	61	Palatine tonsil (2); BOT (1); oropharynx NOS (1)	K (3); NK (1)	4/4 (100%)	55	9 (all cases positive)
HRHPV only n=19 cases	M (15)/ F (4)	57	Palatine tonsil (8); oropharynx NOS (5); BOT (3); uvula (1); tonsillar pillar (1)	NK (11); H (6); K (2)	17/19 (89%)	42	0.5 (1/19 case negative)
LRHPV only n=6 cases	M (4)/ F (2)	62	Palatine tonsil (2); tonsillar pillar (2); BOT(1); soft palate (1)	H (5); NK (1)	5/6 (83%)	59	16 (all cases positive)
HRHPV/LRHPV n=3 cases	M (2)/ F (1)	67	Palatine tonsil (1); BOT (1); oropharynx NOS (1)	NK (2); K (1)	3/3 (100%)	70	13 (1/3 case negative)
HPV-negative n=94 cases (75%)	M (83)/ F (11)	59	Palatine tonsil (21); BOT (20); oropharynx NOS (18); uvula (1); soft palate (14); uvula (7); tonsillar pillar (6)	K (91); NK (2); H (1)	58/94 (62%)	40	7 (23/94 cases negative [24%])

WS HPV, Wide spectrum human papillomavirus; HRHPV, High-risk human papillomavirus; LRHPV, Low-risk human papillomavirus; FOM, floor of the mouth; BOT, base of the tongue; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; K, keratinizing; H, hybrid; NK, non-keratinizing; M, Male; F, Female; Y, years

All HRHPV, LRHPV and HRHPV/LRHPV cases were also positive for wide-spectrum (WS)-HPV probe.

Histopathological grading for all OSCC cases: WD (53%), MD (45%), PD (2%).
Histopathological grading for all OPSCC cases: K (77%), NK (13%), H (10%)
HPV-positive patients, mean age, OSCC vs OPSCC (p=0.147)
For OSCC, HPV-positive vs HPV-negative: Ki-67 (p=0.0289) and Cyclin D1 (p=0.0002).
For OPSCC, HPV-positive vs HPV-negative: Ki-67 (p=0.0338) and Cyclin D1 (p=0.0008).

Table 3. Clinicopathological details of high-risk/low-risk and low-risk human papillomavirus profile in the oral cavity and oropharyngeal squamous cell carcinoma patients.

Oral cavity squamous cell carcinoma patients												
HRHPV/LRHPV	**Gender**	**Age (y)**	**Location**	**Tobbaco smoking**	**Alcohol consumption**	**Histopathological grading**	**Clinical stage at diagnosis**	**TNM**	**Treatment**	**Recurrence**	**Metastasis**	**Outcome**	**Overall Survival (mo) (mean, 21)**
Case 1	M	69	Tongue	no	no	MD	I	T1N0M0	Surg	no	no	Alive	31
Case 2	M	63	Tongue	yes	yes	WD	IVA	T4N0M0	Surg, RTX, Chemo	no	yes	DOD	12
LRHPV only	**Gender**	**Age (y)**	**Location**	**Tobbaco smoking**	**Alcohol consumption**	**Histopathological grading**	**Clinical stage at diagnosis**	**TNM**	**Treatment**	**Recurrence**	**Metastasis**	**Outcome**	**Overall Survival (mo) (mean, 46)**
Case 1	M	66	Tongue	no	no	WD	I	T1N0M0	Surg	yes	no	Alive	34
Case 2	M	64	FOM	yes	yes	WD	II	T2N0M0	Surg, RTX, Chemo	NS	NS	DOD	54
Case 3	M	73	FOM	yes	yes	MD	III	T2N1M0	Surg, RTX	no	no	Alive	50
Oropharyngeal squamous cell carcinoma patients													
HRHPV/LRHPV	**Gender**	**Age (y)**	**Location**	**Tobbaco smoking**	**Alcohol consumption**	**Histopathological grading**	**Clinical stage at diagnosis**	**TNM**	**Treatment**	**Recurrence**	**Metastasis**	**Outcome**	**Overall Survival (mo) (mean, 21)**
Case 1	M	66	BOT	no	no	NK	NS	NS	Surg	NR	NR	DOD	01 checar
Case 2	M	85	Oropharynx NOS	yes	no	NK	IVA	TxN2M0	Surg, RTX	no	no	Alive	28
Case 3	F	50	Palatine tonsil	yes	yes	K	IVA	T4N2M0	Surg, RTX, Chemo	yes	yes	Alive	36
LRHPV	**Gender**	**Age (y)**	**Location**	**Tobbaco smoking**	**Alcohol consumption**	**Histopathological grading**	**Clinical stage at diagnosis**	**TNM**	**Treatment**	**Recurrence**	**Metastasis**	**Outcome**	**Overall Survival (mo) (mean, 29)**
Case 1	F	69	Palatine tonsil	yes	yes	H	IVA	T2N2M0	Surg, RTX, Chemo	no	yes	Alive	104
Case 2	M	75	Pillar tonsillar	yes	yes	H	IVB	T4N3M0	Surg, RTX, Chemo	no	yes	Alive	28

Case 3	M	74	BOT	no	no	H	I	T1	Surg	no	no	DOD	02
Case 4	F	58	Palatine tonsil	yes	yes	H	IVA	TxN2M0	Surg, RTX, Chemo	yes	NS	DOD	07
Case 5	M	55	Pillar tonsillar	yes	yes	H	IVA	T3N2M0	Surg, RTX, Chemo	yes	no	DOD	08
Case 6	M	58	Soft palate	yes	yes	NK	IVA	T4N0M0	Surg, RTX, Chemo	NS	NS	Alive	28

HRHPV, High-risk human papillomavirus; LRHPV, Low-risk human papillomavirus; M, Male; F, Female; Y, Year; DOD, dead of disease; SCC, Squamous cell carcinoma; FOM, floor of the mouth; BOT, base of the tongue; NOS, not otherwise specified; WDSCC, Well differentiated SCC; MDSCC, moderately differentiated SCC; NKSCC, Non-keratinizing SCC; Surg, Surgical resection, RTX, radiotherapy; Chemo, Chemotherapy; mo, Months; NS, not stated.

Overall survival (mean, months): OSCC (HRHPV): 46.5; OPSCC (HRHPV): 28.9; OSCC (HPV-negative): 32.7; OPSCC(HPV-negative): 33.2

5 CONCLUSÃO

Em resumo, além dos casos típicos associados ao ARHPV, nossos resultados mostram que os casos associados ao HPVBR e ao HPVAR/HPVBR (co-infecção) podem ser detectados no CECO e CECOrof, com um aparente impacto na sobrevida. Os mecanismos patogênicos da infecção múltipla por HPV, no entanto, ainda permanecem incertos. Mais estudos são necessários para entender melhor o comportamento clínico-patológico e o impacto prognóstico desses subgrupos de tumores.

Diferentemente do CECO, nossos resultados mostram predominância de imDCs, com perfil de ativação de células imunes, no CECorof. O estatus do HPV parece mostrar associação com a infiltração de DC em ambas as neoplasias, sugerindo respostas imunes antivirais no CECorof associado ao HPV. Estudos futuros com foco em aspectos moleculares para avaliar o status funcional dos subtipos das CDs, bem como seus mecanismos de ativação no microambiente tumoral, provavelmente oferecerão um maior entendimento das estratégias de imunoterapia no tratamento de pacientes com CECCP associado ao HPV e não associado ao HPV.

REFERÊNCIAS*

1. Hübbers CU, Akgül B. HPV and cancer of the oral cavity. Virulence. 2015; 6(3): 244-8.

2. El-Naggar A.K, Takata T. Tumours of the oropharynx: base of tongue, tonsils, adenoids. In: El-Naggar A.K., Chan J.K.C, Grandis J.R, Takata T, Slootweg P. J editors. WHO Classlfication of hed and neck tumours. 4th ed.: Lyon: ARC; 2017. p. 136-8

3. Instituto Nacional de Câncer. Estimativa 2018: incidência de câncer no Brasil. Rio de Janeiro: INCA; 2017. [acesso 14 de jun. 2018]. Disponível em: http://www.inca.gov.br/estimativa/2018/estimativa-2018.pdf.

4. Han N, Zhang Z, Liu S, Ow A, Ruan M, Yang W, Zhang C. Increased tumor-infiltrating plasmacytoid dendritic cells predicts poor prognosis in oral squamous cell carcinoma. Arch Oral Biol. 2017;78: 129-134.

5. Alam H, Bhate AV, Gangadaran P, Sawant SS, Salot S, Sehgal L, et al. Fascin overexpression promotes neoplastic progression in oral squamous cell carcinoma.BMC Cancer. 20;12: 32.

6. Speight PM, Khurram SA, Kujan O. Oral potentially malignant disorders: risk of progression to malignancy. Oral Surg Oral Med Oral Pathol Oral Radiol. 2017. pii: S2212-4403(17): 31248-8.

7. Chi AC, Day TA, Neville BW. Oral cavity and oropharyngeal squamous cell carcinoma-- an update. CA Cancer J Clin. 2015;65(5): 401-21.

8. Montoro JR, Ricz HA, Souza Ld, Livingstone D, Melo DH, Tiveron RC, Mamede RC. Prognostic factors in squamous cell carcinoma of the oral cavity. Braz J Otorhinolaryngol. 2008;74(6): 861-6.

9. Hong AM, Dobbins TA, Lee CS, Jones D, Harnett GB, Armstrong BK, et al. Human papillomavirus predicts outcome in oropharyngeal cancer in patients treated primarily with surgery or radiation therapy. Br J Cancer. 2010;103(10): 1510-7.

10. Vu HL, Sikora AG, Fu S, Kao J. HPV-induced oropharyngeal cancer, immune response and response to therapy. Cancer Lett. 2010;288(2): 149-55.

11. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010; 363(1): 24-35.

* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf

12. Rietbergen MM, Brakenhoff RH, Bloemena E, Witte BI, Snijders PJ, Heideman DA, et al. Human papillomavirus detection and comorbidity: critical issues in selection of patients with oropharyngeal cancer for treatment De-escalation trials. Ann Oncol. 2013;24(11): 2740-5.

13. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Curado MP, Ferlay J, Franceschi S, et al. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. J Clin Oncol. 2013;31(36): 4550-9.

14. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65(2): 87-108.

15. Anantharaman D, Marron M, Lagiou P, Samoli E, Ahrens W, Pohlabeln H, et al. Population attributable risk of tobacco and alcohol for upper aerodigestive tract cancer. Oral Oncol. 2011;47(8): 725-31.

16. Brown JS, Shaw RJ, Bekiroglu F, Rogers SN. Systematic review of the current evidence in the use of postoperative radiotherapy for oral squamous cell carcinoma. Br J Oral Maxillofac Surg. 2012;50(6): 481-9.

17. International Agency for Research on Cancer (IARC). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. List of Classifications by Cancer Site. monographs.iarc.fr/ENG/Classification/index.php. Accessed February 22, 2018.

18. McDowell JD. An overview of epidemiology and common risk factors for oral squamous cell carcinoma. Otolaryngol Clin North Am. 2006;39(2): 277-94.

19. Hoffmannová J, Foltán R, Vlk M, Sipos M, Horká E, Pavlíková G et al. Hemimandibulectomy and therapeutic neck dissection with radiotherapy in the treatment of oral squamous cell carcinoma involving mandible: a critical review of treatment protocol in the years 1994-2004. Int J Oral Maxillofac Surg. 2010;39(6): 561-7.

20. Carnelio S, Rodrigues GS, Shenoy R, Fernandes D. A brief review of common oral premalignant lesions with emphasis on their management and cancer prevention. Indian J Surg. 2011;73(4): 256-61

21. Zur Hausen H. Papillomaviruses in the causation of human cancers-a brief historical account. Virology. 2009;384: 260-265.

22. Zandberg DP, Bhargava R, Badin S, Cullen KJ. The role of human papillomavirus in nongenital cancers. CA Cancer J Clin. 2013;63(1): 57-81.

23. Partlová S, Bouček J, Kloudová K, Lukešová E, Zábrodský M, Grega M, et al. Distinct patterns of intratumoral immune cell infiltrates in patients with HPV-associated compared to non-virally induced head and neck squamous cell carcinoma. Oncoimmunology. 2015;4(1): e965570.

24. Lechner A, Schlößer H, Rothschild SI, Thelen M, Reuter S, Zentis P, et al. Characterization of tumor-associated T-lymphocyte subsets and immune checkpoint molecules in head and neck squamous cell carcinoma. Oncotarget. 2017;8(27): 44418-433.

25. Schiffman M, Wentzensen N, Wacholder S, Kinney W, Gage JC, Castle PE. Human papillomavirus testing in the prevention of cervical cancer. J Natl Cancer Inst. 2011;103: 368-383.

26. Deschler DG, Richmon JD, Khariwala SS, Ferris RL, Wang MB. The "new" head and neck cancer patient-young, nonsmoker, nondrinker, and HPV positive: evaluation. Otolaryngol Head Neck Surg. 2014;151(3): 375-80.

27. Bagan J, Sarrion G, Jimenez Y. Oral cancer: clinical features. Oral Oncol. 2010; 46(6):414-7.

28. Dantas TS, de Barros Silva PG, Sousa EF, da Cunha Mdo P, de Aguiar AS, Costa FW, et al. Influence of Educational Level, Stage, and Histological Type on Survival of Oral Cancer in a Brazilian Population: A Retrospective Study of 10 Years Observation. Medicine (Baltimore). 2016 Jan;95(3):e2314.

29. Bewley AF, Farwell DG. Oral leukoplakia and oral cavity squamous cell carcinoma. Clin Dermatol. 2017 Sep - Oct;35(5):461-467.

30. Fujimaki M, Fukumura Y, Mitani K, Kurisaki A, Yokoyama J, Ikeda K, Yao T. Histological subtypes and characteristic structures of HPV-associated oropharyngeal carcinoma: study with Japanese cases. Diagn Pathol. 2013 Dec 19;8:211.

31. Westra WH, Lewis JS Jr. Update from the 4th Edition of the World Health Organization Classification of Head and Neck Tumours: Oropharynx. Head Neck Pathol. 2017;11(1): 41-47.

32. Ndiaye C, Mena M, Alemany L, Arbyn M, Castellsagué X, Laporte L, Bosch FX, de Sanjosé S, Trottier H. HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. Lancet Oncol. 2014;15(12): 1319-31.

33. Li W, Thompson CH, Xin D, Cossart YE, O'Brien CJ, McNeil EB, Gao K, Scolyer RA, Rose BR. Absence of human papillomavirus in tonsillar squamous cell carcinomas from Chinese patients. Am J Pathol. 2003;163(6): 2185-9.

34. Ribeiro KB, Levi JE, Pawlita M, Koifman S, Matos E, Eluf-Neto J, et al. Low human papillomavirus prevalence in head and neck cancer: results from two large case-control studies in high-incidence regions. Int J Epidemiol. 2011 ;40(2): 489-502.

35. Piña AR, Jimenez LS, Mariano FV, de Andrade BA, Carlos R, Altemani A, de Almeida OP. Human papillomavirus in tonsillar squamous cell carcinomas from Guatemala and Brazil. Oral Surg Oral Med Oral Pathol Oral Radiol. 2016;121(4): 412-8.

36. Matos LL, Miranda GA, Cernea CR. Prevalence of oral and oropharyngeal human papillomavirus infection in Brazilian population studies: a systematic review. Braz J Otorhinolaryngol. 2015;81(5): 554-67.

37. De Cicco R, de Melo Menezes R, Nicolau UR, Pinto CAL, Villa LL, Kowalski LP. Impact of human papillomavirus status on survival and recurrence in a geographic region with a low prevalence of HPV-related cancer: A retrospective cohort study. Head Neck. 2020;42(1): 93-102.

38. Nopmaneepaisarn T, Tangjaturonrasme N, Rawangban W, Vinayanuwattikun C, Keelawat S, Bychkov A. Low prevalence of p16-positive HPV-related head-neck cancers in Thailand: tertiary referral center experience. BMC Cancer. 2019;19(1): 1050.

39. Szkaradkiewicz A, Kruk-Zagajewska A, Wal M, Jopek A, Wierzbicka M, Kuch A. Epstein-Barr virus and human papillomavirus infections and oropharyngeal squamous cell carcinomas. Clin Exp Med. 2002;2(3): 137-41.

40. Huang L, Baban B, Johnson BA 3rd, Mellor AL. Dendritic cells, indoleamine 2,3 dioxygenase and acquired immune privilege. Int Rev Immunol. 2010;29(2): 133-55.

41. Cheraghlou S, Yu PK, Otremba MD, Park HS, Bhatia A, Zogg CK, et al. Treatment deintensification in human papillomavirus-positive oropharynx cancer: Outcomes from the National Cancer Data Base. Cancer. 2018;124(4): 717-726.

42. Carvalho AL, Singh B, Spiro RH, Kowalski LP, Shah JP. Cancer of the oral cavity: a comparison between institutions in a developing and a developed nation. Head Neck. 2004;26(1): 31-8.

43. Dios PD, Lestón JS. Oral cancer pain. Oral Oncol. 2010 Jun;46(6):448-51.

44. Lindel K, Beer KT, Laissue J, Greiner RH, Aebersold DM. Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. Cancer. 2001 Aug 15;92(4):805-13.

45. Yao M, Chang K, Funk GF, Lu H, Tan H, Wacha J, Dornfeld KJ, Buatti JM. The failure patterns of oral cavity squamous cell carcinoma after intensity-modulated radiotherapy-the university of iowa experience. Int J Radiat Oncol Biol Phys. 2007;67(5): 1332-41.

46. Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. J Clin Oncol. 2009;27:1992–1998.

47. Bernier J, Domenge C, Ozsahin M, Matuszewska K, Lefèbvre JL, Greiner RH, et al. Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. N Engl J Med. 2004;350(19): 1945-52.

48. Spanos WC, Nowicki P, Lee DW, Hoover A, Hostager B, Gupta A, et al. Immune response during therapy with cisplatin or radiation for human papillomavirus-related head and neck cancer. Arch Otolaryngol Head Neck Surg 2009;135: 1137-46

49. Cooper JS, Pajak TF, Forastiere AA, Jacobs J, Campbell BH, Saxman SB, et al. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. N Engl J Med. 2004;350(19): 1937-44.

50. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144: 646–74.

51. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science. 2006;313(5795): 1960-4.

52. Hong Y, Manoharan I, Suryawanshi A, Majumdar T, Angus-Hill ML, Koni PA, et al. β-catenin promotes regulatory T-cell responses in tumors by inducing vitamin A metabolism in dendritic cells. Cancer Res. 2015;75(4): 656-665.

53. Genin M, Clement F, Fattaccioli A, Raes M, Michiels C. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. BMC Cancer. 2015;15: 577.

54. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol. 2002;169(5): 2756-61.

55. Sutmuller RP, van Duivenvoorde LM, van Elsas A, Schumacher TN, Wildenberg ME, Allison JP, et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med. 2001;194(6): 823-32.

56. Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D, Zhang A, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Invest. 2005;115(12): 3623-33.

57. Whiteside TL. Down-regulation of zeta-chain expression in T cells: a biomarker of prognosis in cancer? Cancer Immunol Immunother. 2004; 53:865–78.

58. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol. 2000;18: 767-811.

59. Barrett AW, Cruchley AT, Williams DM. Oral mucosal Langerhans' cells. Crit Rev Oral Biol Med. 1996;7(1): 36-58.

60. Steinman RM, Banchereau J. Taking dendritic cells into medicine. Nature. 2007;449(7161): 419-26.

61. Steinman RM, Idoyaga J. Features of the dendritic cell lineage. Immunol Rev. 2010;234(1): 5-17.

62. Lanzavecchia A, Sallusto F. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. Science. 2000;290(5489): 92–7.

63. Adema GJ. Dendritic cells from bench to bedside and back. Immunol Lett. 2009;122(2): 128-30

64. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology 2007; 121: 1–14.

65. Jardim JF, Gondak R, Galvis MM, Pinto CAL, Kowalski LP. A decreased peritumoral CD1a+ cell number predicts a worse prognosis in oral squamous cellcarcinoma. Histopathology. 2018;72(6): 905-913.

66. Aramaki O, Chalermsarp N, Otsuki M, Tagami J, Azuma M. Differential expression of co-signal molecules and migratory properties in four distinct subsets of migratory dendritic cells from the oral mucosa. Biochem Biophys Res Commun. 2011;413(3): 407-13

67. Huang SH, Xu W, Waldron J, Siu L, Shen X, Tong L, et al. Refining American Joint Committee on Cancer/Union for International Cancer Control TNM stage and prognostic groups for human papillomavirus-related oropharyngeal carcinomas. J Clin Oncol. 2015;33: 836-845.

68. Mascarell L, Lombardi V, Louise A, Saint-Lu N, Chabre H, Moussu H, et al. Oral dendritic cells mediate antigen-specific tolerance by stimulating TH1 and regulatory CD4+ T cells. J Allergy Clin Immunol. 2008;122(3): 603-9.

69. Lechmann M, Shuman N, Wakeham A, Mark TW. The CD83 reporter mouse elucidates the activity of the CD83 promoter in B, T, and dendritic cell populations in vivo. Proc Natl Acad Sci USA. 2008;105(33): 11887-92.

70. Chalermsarp N, Azuma M. Identification of three distinct subsets of migrating dendritic cells from oral mucosa within the regional lymph nodes. Immunology. 2009;127(4): 558- 66.

71. Rao SK, Pavicevic Z, Du Z, Kim JG, Fan M, Jiao Y, et al. Pro-inflammatory genes as biomarkers and therapeutic targets in oral squamous cell carcinoma. Biol Chem. 2010;285(42): 32512-21.

72. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 2013;31: 563–604.

73. Bennaceur K, Chapman J, Brikci-Nigassa L, Sanhadji K, Touraine JL, Portoukalian J. Dendritic cells dysfunction in tumor environment. Cancer Lett. 2008; 272; 186–196.

74. Costa NL, Gonçalves AS, Martins AF, Arantes DA, Silva TA, Batista AC. Characterization of dendritic cells in lip and oral cavity squamous cell carcinoma. J Oral Pathol Med. 2016 Jul;45(6): 418-24.

75. Nguyen N, Bellile E, Thomas D, McHugh J, Rozek L, Virani S, et al. Tumor infiltrating lymphocytes and survival in patients with head and neck squamous cell carcinoma. Head Neck. 2016 Jul;38(7): 1074-84.

76. Kindt N, Descamps G, Seminerio I, Bellier J, Lechien JR, Pottier C, et al. Langerhans cell number is a strong and independent prognostic factor for head and neck squamous cell carcinomas. Oral Oncol. 2016;62: 1-10.

77. Goldman SA, Baker E, Weyant RJ, Clarke MR, Myers JN, Lotze MT. Peritumoral CD1a-positive dendritic cells are associated with improved survival in patients with tongue carcinoma. Arch Otolaryngol Head Neck Surg. 1998;124(6): 641-6.

78. Gallo O, Bianchi S, Giannini A, Gallina E, Libonati GA, Fini-Storchi O. Correlations between histopathological and biological findings in nasopharyngeal carcinoma and its prognostic significance. Laryngoscope. 1991;101(5): 487-93.

79. O'Donnell RK, Mick R, Feldman M, Hino S, Wang Y, Brose MS, Muschel RJ. Distribution of dendritic cell subtypes in primary oral squamous cell carcinoma is inconsistent with a functional response. Cancer Lett. 2007;255(1): 145-52.

80. Léon JE, Dos Santos JL, Ribeiro-Silva A, Chahud F, Almeida LKY, Bufalino A, Almeida LY. Submucosal dendritic cells in potentially malignant disorders and squamous cell carcinoma of the oral cavity and lip. Oral Surg Oral Med Oral Pathol Oral Radiol. 2017 Aug 124;supl(2):130.

81. Pellicioli ACA, Bingle L, Farthing P, Lopes MA, Martins MD, Vargas PA. Immunosurveillance profile of oral squamous cell carcinoma and oral epithelial dysplasia through dendritic and T-cell analysis. J Oral Pathol Med. 2017 Nov;46(10):928-933.

82. Abolhalaj M, Askmyr D, Sakellariou CA, Lundberg K, Greiff L, Lindstedt M. Profiling dendritic cell subsets in head and neck squamous cell tonsillar cancer and benign tonsils. Sci Rep. 2018;8(1): 8030.

83. Gomes JO, de Vasconcelos Carvalho M, Fonseca FP, Gondak RO, Lopes MA, Vargas PA. CD1a+ and CD83+ Langerhans cells are reduced in lower lip squamous cell carcinoma. J Oral Pathol Med. 2016;45(6): 433-9.

ANEXO A – Aprovação do comitê de ética em pesquisa (CEP)

USP - HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DE RIBEIRÃO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Caracterização imunoistoquímica comparativa de subgrupos de células dendríticas e oncogênese viral no carcinoma espinocelular oral e orofaríngeo

Pesquisador: Jorge Esquiche León

Área Temática:

Versão: 1

CAAE: 99417018.4.0000.5440

Instituição Proponente: UNIVERSIDADE DE SAO PAULO

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.947.109

Apresentação do Projeto:

O carcinoma espinocelular (CEC) de cabeça e pescoço (CECCP) é a quinta malignidade mais comum nessa região. Recentes estudos enfatizam o CEC oral (CECO) como uma entidade distinta do CEC de orofaringe (CECorof), com este último apresentando melhor prognóstico e estreitamente associado com oncogênese viral. A etiologia do CECCP é considerada multifatorial; porém, o CECO está intimamente relacionado com abuso do tabaco e álcool, enquanto o CECorof está frequentemente associado com infecção pelo papilomavírus humano (HPV). As células dendríticas (CDs), são importantes células do sistema imune as quais regulam repostas imunes, incluindo aquelas vinculadas à tumorigênese, estabelecendo conexão entre o sistema imune inato e adaptativo. Estão divididas em dois grupos: mieloides (CDmi) e plasmocitóides (CDp), incluindo estágios imaturos (CDim) e maduros (CDm). As CDm são responsáveis por iniciar mecanismos imunológicos protetores contra neoplasias e agentes estranhos, incluindo vírus, enquanto que as CDim induzem a tolerância imunológica. O objetivo do nosso estudo será analisar comparativamente, por imunoistoquímica (IQ) e hibridização in situ (HIS), a infiltração de CDmi e CDp, incluindo subgrupos de CDim e CDm, no interior do CECO (n=100) e CECorof (n=50), bem como análise da oncogênese viral (HPV e vírus Epstein-Barr [VEB]), focando associações clinicopatológicas, fatores prognósticos e terapêuticos. Nossos resultados visam contribuir com a elucidação da patogênese do CECO e CECorof e determinar se diferenças na infiltração de subgrupos de CDs, incluindo seus estágios de ativação, em correlação com a oncogênese viral,

Endereço: CAMPUS UNIVERSITÁRIO
Bairro: MONTE ALEGRE **CEP:** 14.048-900
UF: SP **Município:** RIBEIRAO PRETO
Telefone: (16)3602-2228 **Fax:** (16)3633-1144 **E-mail:** cep@hcrp.usp.br

podem explicar o comportamento biológico destes tumores.

Objetivo da Pesquisa:

O objetivo do presente estudo será analisar comparativamente, utilizando um amplo painel Imunoistoquímico, suportado por Hibridização in situ, os diversos eventos celulares mediados por células dendríticas e a oncogênese viral através de associações clinicopatológicas, focando fatores prognósticos e terapêuticos.

Avaliação dos Riscos e Benefícios:

Riscos: O estudo será realizado em blocos parafinados de amostras que já foram excisadas como parte do tratamento realizado no paciente. Os espécimes serão devidamente armazenados em local adequado identificados por códigos, garantindo a preservação do material e seu anonimato visandodiminuir minimizar os riscos. O material ficará sob a guarda do Prof. Dr. Jorge Esquiche León e do Prof. Dr. Alfredo Ribeiro da Silva, os quais ficarão responsáveis pela autorização do uso do material.

Benefícios: Estudos desta natureza são importantes porque as associações clinico-patologicas que serão realizadas podem esclarecer o papel e participação do sistema imune (células dendríticas) na patogênese destes tumores, focando fatores terapêuticos e prognósticos para o paciente.

Comentários e Considerações sobre a Pesquisa:

Será realizado um estudo o qual o número total de blocos parafinados de carcinoma espinocelular oral e orofaríngeo será levantado no período de Março/2018 a Setembro/2018, as amostras do nosso estudo são pertencentes ao departamento de Patologia do HCFMUSP-RP. Serão feitos cortes histológicos (3µm de espessura) obtidos de tecidos fixados em formol e emblocados em parafina, oriundos de biópsias excisionais realizadas previamente e independentemente da pesquisa. Sendo um estudo retrospectivo e baseado na análise de espécimes de arquivo, não haverá contato direto com os pacientes. Devido ao período relativamente longo coberto pelo critério de seleção das amostras e pelo tipo de material, não será possível localizar os voluntários e abordá-los para aplicar o termo de consentimento livre e esclarecido (TCLE). Todos os blocos serão devidamente identificados e a identidade dos pacientes será preservada. Os blocos passarão novamente por revisão histológica e após isso será realizado a técnica da construção dos microarranjos (TMAs) para realização da técnica de imunoistoquímica com os devidos anticorpos. Em um segundo momento para identificação do envolvimento viral nessas amostras será realizada a técnica de Hibridização in situ para Papilomavírus Humano e Vírus Epstein-Barr. A análise estatística será feita utilizando um pacote estatístico previamente definido, assim como testes estatísticos

devidamente selecionados, adotando um nível de significância de p<0,05.

Considerações sobre os Termos de apresentação obrigatória:

Os documentos obrigatórios foram devidamente apresentados. Propõe dispensa do TCLE sendo um estudo retrospectivo e baseado na análise de espécimes de arquivo, não haverá contato direto com os pacientes. Devido ao período relativamente longo coberto pelo critério de seleção das amostras e pelo tipo de material, não será possível localizar os voluntários e abordá-los para aplicar o termo de consentimento livre e esclarecido (TCLE).

Recomendações:

não se aplica

Conclusões ou Pendências e Lista de Inadequações:

Diante do exposto e à luz da Resolução CNS 466/2012, o projeto de pesquisa, assim como a solicitação de dispensa de aplicação do Termo de Consentimento Livre e Esclarecido, podem ser enquadrados na categoria APROVADO.

Considerações Finais a critério do CEP:

Projeto Aprovado: Tendo em vista a legislação vigente, devem ser encaminhados ao CEP, relatórios parciais anuais referentes ao andamento da pesquisa e relatório final ao término do trabalho. Qualquer modificação do projeto original deve ser apresentada a este CEP em nova versão, de forma objetiva e com justificativas, para nova apreciação.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1175693.pdf	25/09/2018 16:53:53		Aceito
Outros	Formulario_SAM.pdf	25/09/2018 16:45:18	HEITOR ALBERGONI DA	Aceito
Outros	autorizacao_laboratorio_histopatologia.pdf	25/09/2018 16:42:39	HEITOR ALBERGONI DA	Aceito
Outros	Orcamento_detalhado.pdf	25/09/2018 16:37:36	HEITOR ALBERGONI DA	Aceito
Outros	Cronograma.pdf	25/09/2018 16:37:03	HEITOR ALBERGONI DA	Aceito
Outros	Documento_UPC.pdf	25/09/2018 16:27:49	HEITOR ALBERGONI DA	Aceito
TCLE / Termos de Assentimento /	TCLE.pdf	25/09/2018 16:26:09	HEITOR ALBERGONI DA	Aceito

Justificativa de Ausência	TCLE.pdf	25/09/2018 16:26:09	HEITOR ALBERGONI DA	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_Detalhado.pdf	25/09/2018 16:19:42	HEITOR ALBERGONI DA SILVEIRA	Aceito
Folha de Rosto	Folha_de_Rosto_.pdf	25/09/2018 16:19:22	HEITOR ALBERGONI DA	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RIBEIRAO PRETO, 08 de Outubro de 2018

Assinado por:
MARCIA GUIMARÃES VILLANOVA
(Coordenador(a))

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DE RIBEIRÃO PRETO DA UNIVERSIDADE DE SÃO PAULO

FMRP-USP RIBEIRÃO PRETO

OFÍCIO Nº 2854/2018
CEP/MGV

RIBEIRÃO PRETO, 11 DE OUTUBRO DE 2018

PREZADOS SENHORES,

O TRABALHO INTITULADO **"CARACTERIZAÇÃO IMUNOISTOQUÍMICA COMPARATIVA DE SUBGRUPOS DE CÉLULAS DENDRÍTICAS E ONCOGÊNESE VIRAL NO CARCINOMA ESPINOCELULAR ORAL E OROFARÍNGEO"**, FOI ANALISADO PELO COMITÊ DE ÉTICA EM PESQUISA, EM SUA 478ª REUNIÃO ORDINÁRIA REALIZADA EM 08/10/2018 E ENQUADRADO NA CATEGORIA: APROVADO, BEM COMO A SOLICITAÇÃO DE DISPENSA DO TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO, DE ACORDO COM O PROCESSO HCRP Nº 11582/2018.

ESTE COMITÊ SEGUE INTEGRALMENTE A RESOLUÇÃO Nº 466/12 CNS/MS.

LEMBRAMOS QUE DEVEM SER APRESENTADOS A ESTE CEP, O RELATÓRIO PARCIAL E O RELATÓRIO FINAL DA PESQUISA.

ATENCIOSAMENTE.

DRª. MARCIA GUIMARÃES VILLANOVA
COORDENADORA DO COMITÊ DE ÉTICA EM PESQUISA DO HCRP E DA FMRP-USP

Ilustríssimos Senhores
JORGE ESQUICHE LEÓN
PROF.DR.ALFREDO RIBEIRO DA SILVA(ORIENTADOR)
Depto. de Patologia e Medicina Legal

Não autorizo a publicação deste trabalho pelo prazo de 2 anos após a data de defesa

Araraquara, 19 de Fevereiro de 2020.

Heitor Albergoni da Silveia