FACULDADE DE ODONTOLOGIA

ASSOCIAÇÃO ENTRE INFECÇÃO PELOS VÍRUS HPV E EBV E NEOPLASIAS MALIGNAS DE BOCA

ANÁLISE HISTOMORFOMÉTRICA

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2015

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

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ASSOCIATION BETWEEN HPV AND EBV INFECTION AND ORAL CANCER - HISTOMORPHOMETRIC ANALYSIS -

Porto Alegre

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre pelo Programa de Pós-Graduação em Odontologia, Área de Concentração: Estomatologia Clínica

Orientadora: Profª. Drª. Karen Cherubini

Porto Alegre

2015

Dados Internacionais de Catalogação na Publicação (CIP)

J82 Jornada, Vanessa Chidiac
Associação entre infecção pelo vírus HPV e EBV e neoplasias malignas de boca: análise histomorfométrica / Vanessa Chidiac Jornada. – Porto Alegre, 2015.
104 f.: il.

Dissertação (Mestrado em Odontologia) – Faculdade de Odontologia, PUCRS.
Orientação: Profª. Drª. Karen Cherubini.

1. Odontologia. 2. Estomatologia clínica. 3. Neoplasias bucais. 4. Carcinoma oral. 5. Papillomavirus Humano. 6. Epstein-Barr Vírus. I. Cherubini, Karen. II. Título.

CDD 616.99431

Aline M. Debastiani
Bibliotecária - CRB 10/2199

Feliz aquele que transfere o que sabe e aprende o que ensina.

Cora Coralina (1889-1985)

Dedicatória

Aos meus pais, Marcelo e Valéria, e à minha avó, Soad,

por todo o exemplo, amor, suporte e incentivo.

Agradecimentos

A Deus, pelo dom da vida.

Aos meus pais, Marcelo e Valéria, por todo amor, carinho, suporte e dedicação. Esta conquista só foi possível graças ao incentivo e apoio de vocês.

A todos os meus familiares e amigos, obrigada por serem os melhores! O carinho, a cumplicidade, o apoio e o incentivo de vocês foram fundamentais em todas as conquistas da minha vida.

À minha orientadora, Profa. Dra. Karen Cherubini, pela dedicação a este trabalho e a todos que conduz, sempre com extrema excelência e sabedoria. Muito obrigada por todo conhecimento que me passaste e que adquirimos juntas.

Às professoras da Estomatologia, Fernanda Gonçalves Salum, Liliane Soares Yurgel e Maria Antonia Zancanaro de Figueiredo, por todos os ensinamentos, profissionais e pessoais, compartilhados durante essa caminhada.

À Coordenadora do Programa de Pós-Graduação em Odontologia da PUCRS, Profa. Dra. Ana Maria Spohr, por todo seu empenho.

Aos demais professores do Programa de Pós-Graduação em Odontologia da PUCRS, pelos inestimáveis ensinamentos transmitidos.

Aos funcionários da Secretaria de Pós-Graduação da Faculdade de Odontologia da PUCRS, Davenir, Gabriel, Kléber e Vanessa, pela paciência, dedicação e competência despendidas ao nos atender.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo financiamento deste Curso de Pós-graduação.

Ao técnico do Laboratório de Anatomia Patológica e Citopatologia do Hospital São Lucas da PUCRS, Tiago Giuliani Lopes, pela dedicação, competência e persistência na confecção das lâminas histológicas.

Ao Prof. Dr.Vinicius Duval da Silva, Chefe do Laboratório de Anatomia Patológica e Citopatologia do Hospital São Lucas da PUCRS, por disponibilizar as dependências do Laboratório para a execução desta pesquisa.

Aos meus colegas e amigos, Letícia Cuba, Monique Acauan, Clarissa Medeiros, Renata Boff, Felipe Martins, Jamil Saleh, Juliana Spanemberg, Lisiane Cândido, Maria Noel Petruzzi, Ruchielli Borghetti, Luiza de Almeida, Francisco Medella, Ricardo Paiva, Juliana Jasper e Valesca Koth, pela convivência maravilhosa nesses dois anos. Foi ótimo ter compartilhado dúvidas, aprendizado, conquistas e alegrias com vocês.

Às funcionárias do Serviço de Estomatologia do Hospital São Lucas da PUCRS, Cristiane Carlotto e Márcia Luísa Rollsing pela eficiência e dedicação.

Aos pacientes do Serviço de Estomatologia do Hospital São Lucas da PUCRS, por toda confiança em nós depositada, possibilitando assim um imenso aprendizado.

A todos que, de alguma forma, contribuíram para a concretização deste sonho! Muito obrigada por tudo.

Resumo

RESUMO

Os tumores malignos da região de cabeça e pescoço constituem um grupo de doenças que afeta a face e o trato aerodigestivo superior. O diagnóstico precoce dessas neoplasias e de seus fatores etiológicos é de extrema importância para a sobrevida e para o tratamento do paciente. Nas últimas décadas, a relação de causa e efeito entre certos vírus e tumores malignos tem sido amplamente aceita. Recentemente, foi relatado o papel do *Human papillomavirus* (HPV) e do *Epstein-Barr virus* (EBV) na patogenia do carcinoma espinocelular de orofaringe, no carcinoma indiferenciado de nasofaringe e em linfomas, sendo ainda controverso o papel desses agentes virais no carcinoma de células escamosas oral (CCEO). O presente estudo teve por objetivo investigar, por meio de exame imunoistoquímico, os vírus HPV e EBV, bem como os marcadores Ki-67 e p53 em carcinomas e linfomas orais considerando fatores clínicos e histológicos. Espécimes de biópsia arquivados em blocos de parafina e prontuários de pacientes portadores de lesões orais de CCEO, linfoma e hiperplasia fibroepitelial foram distribuídos em cinco grupos: (1) 16 amostras de CCEO grau I (bem diferenciado); (2) 16 amostras de CCEO grau II (moderadamente diferenciado); (3) 19 amostras de CCEO grau III (pobremente diferenciado); (4) 14 amostras de linfoma não-Hodgkin e (5) 19 amostras de hiperplasia fibroepitelial. Os espécimes de biópsia foram submetidos a processamento imunoistoquímico para HPV, EBV, p53 e Ki-67. Dados clínicos referentes a idade e sexo dos pacientes, tamanho e duração da lesão, comorbidades e hábitos foram coletados dos prontuários. A expressão dos marcadores foi analisada de acordo com os aspectos clínicos e grau histológico dos tumores. Não houve associação entre HPV e EBV com os tumores analisados. A expressão do Ki-67 foi significativamente menor no grupo hiperplasia fibroepitelial, não havendo outras diferenças significativas entre os demais grupos. Os linfomas e as hiperplasias fibroepiteliais exibiram expressão de p53 significativamente menor que os carcinomas, sem outras significâncias. CCEO grau II e III foram associados ao sexo masculino e ao tabagismo, enquanto o CCEO grau III foi também associado ao consumo de álcool. Não houve associação dos tumores avaliados com chimarrão ou comorbidades. Houve correlação negativa entre duração e tamanho da lesão, bem como entre tamanho da lesão e HPV. EBV e HPV exibiram correlação positiva, assim como o Ki-67 foi correlacionado ao tamanho da lesão e à p53, que por sua vez exibiu correlação positiva com o HPV.

Conclusão: Os resultados do presente estudo não confirmam a associação do HPV e do EBV com CCEO e linfoma oral. Tabagismo e consumo de álcool foram associados com CCEO graus II e III, mas não com o grau I. As lesões orais analisadas exibiram correlação positiva entre HPV e EBV, o que sugere a ocorrência de coinfecção.

Palavras-chave: HPV, EBV, carcinoma de células escamosas oral, linfoma, oncogênese viral

Summary

SUMMARY

Head and neck cancer represents a group of diseases affecting the face and upper aerodigestive tract. Early diagnosis of these neoplasms and their etiologic factors is crucial for patient's survival and treatment. In the last decades, cause-and-effect relationship between some viruses and malignancies has been widely accepted. Recently, the role of *Human papillomavirus* (HPV) and *Epstein-Barr virus* (EBV) has been shown in the pathogenesis of oropharyngeal carcinoma, undifferentiated nasopharyngeal carcinoma and lymphomas. Nevertheless, the role of such viral agents in oral squamous cell carcinoma (OSCC) is still controversial. This study aimed to investigate HPV, EBV, p53 and Ki-67 in OSCC and oral lymphoma, considering clinical and histological features. The sample was composed of archived material (medical records and paraffin blocks of biopsied specimens) from patients with OSCC, oral lymphoma, and oral traumatic fibrous hyperplasia. The biopsied specimens and medical records were allocated into five groups: (1) 16 samples from patients with OSCC grade I (well-differentiated); (2) 16 samples from patients with OSCC grade II (moderately-differentiated); (3) 19 samples from patients with OSCC grade III (poorly-differentiated); (4) 14 samples from patients with oral non-Hodgkin lymphoma; and (5) 19 samples from patients with oral traumatic fibrous hyperplasia. Biopsied specimens were analyzed for HPV, EBV, p53 and Ki-67 expression by immunohistochemistry, and clinical data concerning age and gender of the patients, size and duration of the lesion as well as habits and comorbidities were collected. Marker expression was evaluated according to clinical features and histological grade of the tumors. There was no association of HPV and EBV with the tumors analyzed. Ki-67 expression was significantly lower in the fibrous hyperplasia group with no other significant differences. p53 expression was significantly lower in the lymphoma and fibrous hyperplasia groups than the OSCC groups, with no other significant differences. OSCC grade II and III were associated with male gender and tobacco smoking, while grade III was also associated with alcohol consumption. There was no association of the tumors with either *chimarrão* or comorbidities. Duration of the lesion was inversely correlated to lesion size, which in turn was inversely correlated to HPV. EBV and HPV were positively correlated to each other, as was Ki-67 to lesion size and p53, which was correlated to HPV.

Conclusion: The results do not support an association of HPV and EBV with OSCC and oral lymphomas. Positive correlation between HPV and EBV in the oral lesions analyzed suggested co-infection.

Key words: HPV, EBV, oral squamous cell carcinoma, lymphoma, viral oncogenesis

Sumário

SUMÁRIO

Introdução

1 INTRODUÇÃO

A maioria dos tumores malignos de cabeça e pescoço é composta por carcinomas de células escamosas que se originam da mucosa (95%), sendo boa parte deles originários da mucosa oral. Na cavidade oral, esses tumores acometem diversos sítios anatômicos, incluindo língua, assoalho de boca, palato, mucosa jugal e gengiva e vêm, na última década, exibindo um importante incremento em sua incidência (BAO-HNS, 2002). Os fatores etiológicos do carcinoma da cavidade oral estão bem estabelecidos na literatura, sendo os principais fatores extrínsecos o tabagismo, o etilismo e a radiação ultravioleta e, entre os fatores intrínsecos estão desnutrição, deficiências vitamínicas e desregulação genética, que se traduz pelo desequilíbrio entre oncogenes e genes supressores tumorais (Tsantoulis *et al.*, 2007; Neville *et al.*, 2009; Wilkey *et al.*, 2009). Tabagismo e etilismo, especialmente quando combinados, constituem os principais fatores extrínsecos associados ao desenvolvimento desses tumores (Du *et al.,* 2000; Figuero-Ruiz *et al.,* 2004; Du *et al.,* 2007; Curado; Hashibe, 2009; Benowitz *et al.,* 2012; Gupta; Metgud, 2013). Estudos têm demonstrado também o papel de diversos vírus na etiologia de tumores malignos, sendo os mais estudados o vírus do papiloma humano (HPV) e o vírus Epstein-Barr (EBV), que têm sido associados ao desenvolvimento, à progressão, à agressividade, à invasão local e às metástases desses tumores (Gao; Zheng, 2011).

O EBV é um membro da família *Herpesviridae* que infecta aproximadamente 90% da população mundial, sendo a infecção, frequentemente, assintomática (Bajaj *et al.*, 2007). Esse vírus entra no hospedeiro humano através da mucosa oral atingindo as células epiteliais da orofaringe, onde ocorre replicação e infecção dos linfócitos B. As células B infectadas não produzem partículas virais infecciosas, mas expressam proteínas virais e caracterizam o estado de latência, sendo fenotipicamente indistinguíveis de células B de memória. Desta forma, essas células são invisíveis à resposta imunológica (Bollard *et al.,*

2012). Apesar de permanecer latente durante toda a vida do indivíduo, em algumas situações o EBV está associado a várias doenças proliferativas benignas e malignas de origem linfoide, tais como mononucleose infecciosa, doença linfoproliferativa pós-transplante (PTLD), linfoma de Burkitt, linfoma de Hodgkin e linfoma não-Hodgkin. Estudos comprovam também o papel significativo do EBV no desenvolvimento do carcinoma gástrico (Rickinson, 2014) e do carcinoma indiferenciado de nasofaringe (Pathmanathan *et al.*, 1995; Rickinson, 2014). Em função de o EBV ter sua contribuição para a transformação maligna de linfócitos B bem estabelecida nesses subtipos de tumores, seu papel oncogênico tem sido amplamente estudado nessas enfermidades e também, mais recentemente, nos carcinomas da cavidade oral (Ammatuna e*t al.*, 2001; Iamaroon e*t al.*, 2004; Laborde e*t al.*, 2010).

A proteína latente de membrana 1 (LMP1) é a chave da carcinogênese associada ao EBV, desempenhando mecanismos complexos de transformação maligna celular, como expressão/ativação de oncogenes; modulação da expressão de genes supressores de tumor; modulação do *checkpoint* G1-S do ciclo celular; indução da expressão de citocinas pró-inflamatórias; indução da transição epitélio-mesênquima (EMT) e modulação celular de micro–RNA´s (miRs). Além disso, a LMP1 confere resistência a apoptose e promove motilidade celular, invasão e metástase (Wang *et al.*, 1985; Dawson *et al*., 2012; Guo *et al.*, 2012; Senyuta e*t al.*, 2014).

Os HPVs, por sua vez, são vírus pequenos, não envelopados, que pertencem à família *Papillomaviridae*. Até o momento, mais de 170 tipos de HPV foram identificados (de Villiers, 2013; Wang *et al*., 2015) e classificados de acordo com seu potencial oncogênico em subtipos de alto risco (integram-se ao DNA celular) e subtipos de baixo risco (forma episssomal). Os HPVs de alto risco são os principais causadores dos carcinomas de colo uterino, anogenitais e, mais recentemente, de orofaringe, enquanto os

de baixo risco estão associados às verrugas mucosas e dérmicas benignas como o condiloma acuminado e o papiloma oral (Campisi *et al.*, 2007). A transmissão do HPV ocorre predominantemente por via sexual, e o seu papel como pré-requisito para o desenvolvimento de câncer do colo uterino foi estabelecido nos anos 80 (Boshart *et al.*, 1984; Dürst *et al.*, 1983). Recentemente, estudos têm demonstrado altos índices de infecção pelo HPV em carcinomas de orofaringe, o que poderia estar associado diretamente ao desenvolvimento e à progressão dessas lesões (Benson *et al.*, 2014). Os subtipos de alto risco, principalmente o HPV 16, apresentam duas importantes oncoproteinas, E6 e E7, que agem sequencialmente no comportamento maligno dos tumores, por meio de mecanismos de inibição de supressores tumorais como as proteínas p53 e de susceptibilidade ao retinoblastoma -pRb (Münger *et al*., 1992; zur Hausen, 2002; Rampias *et al*., 2009; Ghittoni *et al*., 2010; Tommasino *et al*., 2014).

A identificação do papel viral no desenvolvimento dos tumores é de extrema importância para o desenvolvimento e para a otimização de medidas preventivas e terapêuticas como imunização, prevenção e detecção precoce da infecção. Apesar de o HPV já ter sido classificado como pré-requisito ao desenvolvimento de carcinomas do colo uterino (Lingen *et al*., 2013) e de sua associação com o carcinoma de orofaringe parecer consistente (Benson *et al.*, 2014), seu papel na etiopatogênese do carcinoma de células escamosas da cavidade oral permanece controverso (Gillison *et al*., 2000; Lingen *et al*., 2013). Além disso, a infecção simultânea por HPV e EBV tem sido alvo de diversos estudos (Mirzamani *et al*., 2006; Higa *et al*., 2003; Al Moustafa *et al*., 2009) que levantam a hipótese de que a coinfecção por dois ou mais vírus teria papel significativo no desenvolvimento de tumores malignos, o que ainda não foi confirmado (Al Moustafa *et al*., 2009). Desta forma, o presente estudo teve por objetivo investigar a associação entre infecção pelos vírus HPV e EBV e neoplasias malignas de boca, bem como sua correlação

com fatores clínicos e grau de proliferação tumoral. O trabalho é apresentado sob a forma de dois artigos: o artigo 1 consiste em uma revisão da literatura sobre o papel dos vírus HPV e EBV em neoplasias malignas de cabeça e pescoço, enquanto o artigo 2 corresponde ao experimento, que investigou esses vírus em linfomas e carcinomas da cavidade oral.

2 ARTIGO 1

O artigo a seguir intitula-se ***Involvement of Human papillomavirus (HPV) and Epstein-Barr virus (EBV) in head and neck cancer: etiopathogenesis and treatment implications with focus on squamous cell carcinoma*** e foi formatado de acordo com as normas e submetido ao periódico *Archives of Oral Biology* (Anexos A e B).

Involvement of *Human papillomavirus* (HPV) and *Epstein-Barr virus* (EBV) in head and neck cancer: etiopathogenesis and treatment implications with focus on squamous cell carcinoma

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Key words: EBV, HPV, oral squamous cell carcinoma, lymphoma, viral oncogenesis

ABSTRACT

We present here a literature review of the role of *Human papillomavirus* (HPV) and *Epstein-Barr virus* (EBV) in the etiopathogenesis of head and neck cancer and its treatment implications, with focus on squamous cell carcinoma. HPV and EBV have been previously related to the development of carcinoma of the uterine cervix and lymphomas, respectively. In the last decades, studies have reported the oncogenic role of these viruses also in head and neck carcinomas, where EBV is one of the major factors associated with nasopharyngeal carcinoma, and where HPV is related to oropharyngeal squamous cell carcinoma. The interplay of viral proteins with host, especially E6/E7 from HPV, and LMP1 from EBV, is capable of promoting malignancy. Despite the intensive research in this field, the exact mechanisms of viral oncogenesis in head and neck carcinomas still need to be clarified, calling for further studies to achieve new preventive and therapeutic measures for the disease.

INTRODUCTION

Head and neck cancer comprises malignant tumors affecting the face and upper aerodigestive tract, including oral cavity, salivary glands, maxillary bones, facial skin, nasal cavity, paranasal sinuses, pharynx, larynx, and thyroid. About 95% of tumors in this region are squamous cell carcinomas arising from the mucosa.[1] Such neoplasms are strongly associated with environmental and lifestyle risk factors, especially tobacco, alcohol, malnutrition, ultraviolet radiation, chemical products and viral infections.[1-5] In the last decades the cause-and-effect relationship between viruses and tumors, especially malignant ones, has been widely accepted.[1-6] Although at first, it was believed that viruses were associated with the genesis of cancer only in animals,[7] nowadays, a variety of viral agents have been definitely associated with human tumors, including oral ones.[8] Therefore, a viral role has been more and more studied in the

development, progression, aggressiveness, local invasion and metastasis of such tumors.[4,9,10]

Human papillomavirus (HPV)[6,11] and *Epstein-Barr* virus (EBV)[2,4,12] are among the viruses that infect oral tissues and are recognized as oncogenic agents. HPV is clearly associated with carcinoma of the uterine cervix, and its different genotypes are classified according to oncogenic potential as low risk and high risk HPVs.[13] Moreover, the involvement of EBV in the malignant transformation of B lymphocytes has already been established.[14] It has been reported that EBV and HPV can have an oncogenic role also in head and neck squamous cell carcinogenesis.[4] Even though EBV has been associated with nasopharyngeal carcinoma[10] and also more often detected in oral lesions of lichen planus and squamous cell carcinoma, if compared to normal oral epithelium,[14] its influence in the pathogenesis of oral squamous cell carcinomas remains undetermined.[8,14] Also, the same controversy seems to be over the relationship between HPV and such tumors.[8,15]

Considering that there are still unclear aspects about the idea of viruses being an established risk factor for head and neck squamous cell carcinoma,[8,15] we present here a literature review focusing on the role of HPV and EBV in the etiopathogenesis of this cancer and the perspectives this issue raises for its prevention and treatment.

VIRUSES AND TUMORIGENESIS

Viral etiology has been studied in cancer since the beginning of the XXth century after the isolation of an infectious agent which, later on, was recognized as a virus capable of inducing tumors in chickens.[16,17] The idea that specific viral oncogenes could transform normal cells into cancer ones came to light in the 1960s, when the suspicion of Denis Burkitt that the novel childhood African tumor could be linked to some infectious agent was proved with the identification of EBV by the virologists Tony Epstein and Yvonne

Barr.[17] More recently, viral involvement in the development of squamous cell carcinoma has been reported in various studies,[10,18-21] where HPV, EBV and *Herpes simplex* virus type 1 (HSV–1) are the most studied agents and also considered the synergistic viruses most probably involved in human oral carcinogenesis.

Viruses are organisms composed of nucleic acid, DNA or RNA (genome/core), covered by a protein coat (capsid) and an external lipid bilayer containing viral proteins (envelope).[22-25] Some of them such as HPV do not have envelope, where they are formed just by nucleic acid and capsid. Virus size can vary from 10 nm to 300 nm,[24] being generally around or below the limit of the resolution of light microscopy.[26] Because of their constitution, these organisms are obligate intracellular parasites, showing tropism for a specific host cell. Since the cell is infected, the virus can stay in the episomal form or integrate into the cell genome and then start using cell machinery to produce its own proteins and progeny. Virus-host cell interplay, in turn, is capable of interfering with cell proliferation and maturation control mechanisms.[24]

Human oncogenic viruses are defined as necessary but not sufficient to start a neoplasia. Studies suggest that the oncogenic potential of a virus is greater in cells that have already accumulated a certain number of genetic mutations leading to cell cycle deregulation.[27] Still, coinfection with two or more viruses was associated with increased risk of carcinogenesis, including head and neck squamous cell carcinogenesis.[28,29]

The onset of tumors in carriers of tumorigenic viruses is considered a rare event that happens after decades of infection.[27] Some viruses have been shown to be capable of favoring the maintenance of the malignant phenotype and tumor cell progression,[30] as well as contributing with cell mechanisms of escape from both apoptosis and immune surveillance. Tumor initiation events mediated by a virus follow the viral entrance in

genetically intact cells, which can result in replication or latency. Regarding the latter, the oncogenic potential of latent viruses seems low *in vivo*. However, before the silencing of most of virus-specific transcription is accomplished, many viral functions are expressed, which could induce genetic/epigenetic damage in part of the infected population.[27] This can be observed in studies that evidence the interruption or alteration of histone acetyltransferase complex (p300/CBP) activity as an event common to many oncogenic viruses, which suggests that this is a critical event in the initial phases of viral carcinogenesis.[31] Cells surviving such damage could (a) experience virus reactivation, (b) harbor the viral genome in the latent state, or (c) lose it after unequal segregation of their genetic material. In injured cells, latent gene products can represent an effective oncogenic threat if cell caretaker genes are affected. Still, reinfection or reactivation of latent viruses in these cells can result in greater genetic damage, leading to genetic instability, cell immortalization and tumor development.[27]

EBV

EBV was first identified in 1964, and afterwards, many other tumor-associated viruses were reported.[7] EBV, a gammaherpesvirus, is a herpes family member that infects approximately 90% of the world-wide population. Hosts that have been primarily exposed to EBV frequently develop an asymptomatic infection, especially if it occurs in infancy. However, if primary exposure happens during adolescence, the disease called infectious mononucleosis often manifests.[7,9]

EBV infection and latency are better understood by means of the germinal center model (GCM),[32] where the virus is transmitted through the oral route establishing a lytic infection in permissive cells of the oropharynx (squamous epithelial cells and B lymphocytes),[33] which leads to dissemination at high levels in saliva and the throat. The virus gains access to the lymphoid tissue of Waldeyer's ring (tonsils and

adenoids),[34] where it crosses the epithelial barrier and directly infects virgin B cells (naïve) inducing them to proliferate. These cells are then released as memory B cells with latent infection and limited to expressing just viral genome protein EBNA1, or they may not express any viral protein at all. Memory compartment is considered the site of long-term persistence, where virus is quiescent and, therefore, invisible to the immune system.[32] Afterwards, at any time, a small subset of memory B cells with latent infection starts lytic reactivation.[32-35] The result of reactivation can be the release of infective viral particles, which can be downloaded in saliva causing infection dissemination, or infecting new naïve B cells, thus completing the cycle (Fig. 1).[32]

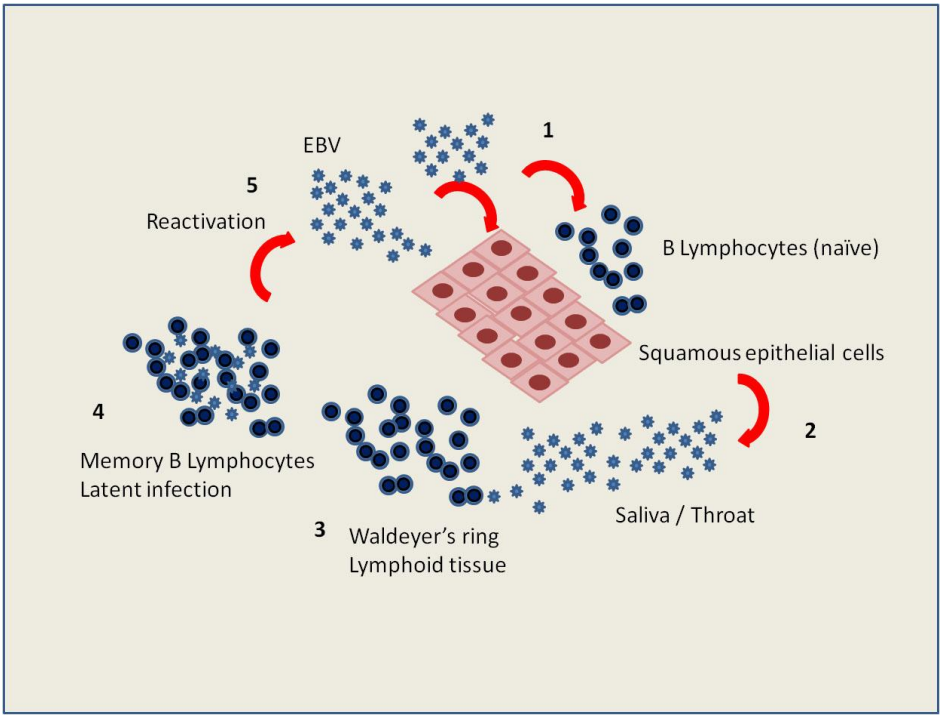

Figure 1 - EBV infection and latency. EBV is transmited by oral route, infecting squamous epithelial cells and B lymphocytes in oropharynx (1), which leads to dissemination at high levels in saliva and throat (2). The virus gains access to lymphoid tissue of Waldeyer's ring, where it crosses epithelial barrier and directly infects naïve B cells, inducing them to proliferate (3). These cells are released as memory B cells with latent infection (4). At any time, a small subset of memory B cells with latent infection starts lytic reactivation, releasing infective viral particles, which can be downloaded in saliva, causing infection dissemination, or infecting new naïve B cells (5)[32]

EBV can be associated with various proliferative disorders of lymphoid origin, such as Burkitt's lymphoma, Hodgkin's disease, post-transplant lymphoproliferative

disease, plasmablastic lymphoma, certain diffuse B cell and T cell lymphomas including T/natural killer cell and immunoangioblastic ones and lymphomatoid granulomatosis as well as.[9,33,36] An association between EBV and lymphomas has been explored for diagnostic and therapeutic purposes. Monitoring EBV DNA in peripheral blood as a marker of EBV-related lymphomas helps to estimate risk and prognosis or to choose the best therapy. Nevertheless, in most cases treatment still does not differ from that used in EBV-negative cases. Current and novel therapies are focused on biological aspects of EBV, in a way to offer future strategies more directed to EBV-positive lymphomas. Besides, vaccines that are in clinical trials seem to be the most promising alternative for treatment or prevention of malignancies related to this virus.[37]

EBV is an oncogenic virus with a proved role in a series of malignant neoplasms,[38] and considering solid tumors, its role is well documented in nasopharyngeal carcinoma and gastric carcinoma.[9] The very high prevalence of EBV infection in nasopharyngeal carcinomas[10] indicates its participation in the etiopathogenesis of such tumors. Although the clear mechanism by which this happens has not yet been explained, it is suggested that it involves a multi-step process over a long period of time with interactions between EBV genes and host genetic alterations in premalignant nasopharyngeal cells.[39] In a study confirming an association between EBV and these tumors, a sample of 20 nasopharyngeal carcinomas showed positivity for EBV-encoded small RNA (EBER) in 19 cases (95%). Thirty-five per cent of them showed marked expression (more than 65% EBER-positive nuclei), 30% showed moderate expression (33 to 65% EBER-positive nuclei), and 35% showed low expression (<33% EBER-positive nuclei).[10]

According to Pathmanathan *et al*.[38] and Higa *et al*.,[40] while the association between undifferentiated nasopharyngeal carcinoma and EBV is well established, other

subtypes of carcinomas still do not have such relationship determined, which reinforces the heterogeneity of etiology in these neoplasms. Anyway, new findings have shown that the virus has a cell infection spectrum wider than previously known, being detected in cells of other tumors, with evidence of its participation in their genesis.[9,33] In this context, the expression of latent genes of EBV has been found in oral squamous cell carcinomas, which suggests the role of EBV in their development or its action along with known risk factors,[41] such as tobacco and alcohol.[9]

Viral proteins involved in EBV-induced carcinogenesis include latency-associated membrane proteins -1 and -2 (LMP-1 and LMP-2) and nuclear antigen-1 to -6 (EBNA 1 to 6).[14,42,43] Nasopharyngeal carcinoma harbors multiple copies of monoclonal episomal EBV and expresses EBNA1, LMP1 and LMP2A viral proteins. The major role of EBNA1 is to maintain episomal EBV, whereas the function of LMP2A is still uncertain, even though it has been attributed to EBV reactivation. LMP1 is a potent oncogene that activates host mechanisms implicated in tumor invasion and suppression of immune responses. It plays an important role in the development of EBV-associated malignant neoplasms and acts through mechanisms such as (1) induction of cytokine expression and resistance to apoptosis and epithelial-mesenchymal transition (EMT); (2) inhibition of tumor-suppressor expression, ensuring dysplastic cell survival; and (3) increase in cell motility, invasion and metastasis.[21,44-46] Among EBV genes, LMP1 deserves more attention because it is the key to EBV-associated carcinogenesis. It is detected at the protein level in up to 65% of nasopharyngeal carcinomas and at transcriptional level in practically all cases.[47]

Immunotherapy for EBV

Studies on immunotherapy have focused on vaccines capable of intensifying immune response mediated by T cells, targeting EBV proteins expressed by tumor cells,

especially EBNAs and LMPs. Another approach consists in infusing patients with EBV-specific autologous cytotoxic T cells expanded *in vitro*. Although very laborious, clinical trials showed that this therapy induced complete response with lesion resolution in some patients with nasopharyngeal carcinoma and Hodgkin's lymphoma. The results suggest that, to be effective against EBV-associated cancer, vaccines need to be capable of inducing a strong T cell-mediated response.[48]

The gp350 vaccine against EBV can prevent or reduce the severity of some conditions related to the virus, either inflammatory or neoplastic. Although perspectives are promising, a vaccine is not yet available for general use. The major challenge is the difficulty of clinical trials being preceded by studies in animals, because of the absence of a satisfactory animal model except subhuman primates.[49] In phase II clinical trials, gp350-based vaccine for EBV reduced infectious mononucleosis rates in EBV-seronegative humans, but it did not affect virus infection rates.[50] Nevertheless, a gp350-based vaccine inducing neutralizing antibodies may not be sufficient for all suggested indications, so there is a need to produce more than one vaccine modality. That is, vaccines inducing T cell response to antigens in tumor cells, especially EBNAs and LMPs, may be needed to prevent or treat malignant neoplasms associated with EBV.[49] Phase I clinical trials showed that, because of its immunogenicity to both TCD4+ and TCD8+ lymphocytes, recombinant modified vaccinia Ankara (MVA-EL) is a potential therapeutic alternative for nasopharyngeal carcinoma.[51,52]

According to some authors,[49,50] there are barriers to EBV vaccine development, such as (1) whether and which viral proteins, besides gp350, would be more effective to prevent mononucleosis or EBV-related malignant neoplasms; (2) difficulties in performing clinical trials to prevent EBV-related tumors because of the lack of good surrogate markers of tumor development and long-term period between EBV primary

infection and development of many EBV+ tumors; (3) lack of immune correlates of protection against EBV infection and diseases in humans and animal models; (4) limitations of animal models; and (5) need for additional information about the economic and societal burden of infection to evaluate cost-benefit ratio of a prophylactic vaccine. Regarding the global impact of malignant and non-malignant EBV-associated diseases, it is extremely important to understand the complete spectrum of immune system control as well as its perturbations to achieve the ideal vaccine.[33,49]

HPV

Human papillomavirus (HPV) is a small non-enveloped virus, which belongs to the family *Papillomaviridae*, a group of small double-stranded DNA viruses (about 8000 bp) that have tropism for the epithelium of the upper respiratory tract, genitals and skin.[53] HPV is mainly associated with cervical cancer, but also with anogenital and head and neck cancers.[54] The role of this virus as a pre-requisite for the development of cervical cancer was established in the 1980s.[55,56] Up to now, about 170 HPV types infecting oral mucosa and genital tract have been identified and classified according to their oncogenic potential[57] in low-risk subtypes (e.g., types 6 and 11), which are associated with benign genital warts; and high-risk subtypes (e.g., 16 and 18), which are the etiological agents of cervical carcinoma.[19] Prevalence of infection with high-risk HPV in tissue samples of uterine cervix cancer has been estimated at 90 to 99%.[13,58-60] Mostly subtypes 16 and 18 and less commonly 31, 33, 35 and 51 are found in approximately 85% of uterine cervix malignancies, including *in situ* and invasive carcinomas.[61]

Molecular aspects and life cycle of HPV

The HPV genome can be divided into three distinct regions: a coding region containing the early genes E1, E2, E4, E5, E6 and E7; a region containing the late genes L1 and L2, which encode respectively the major and minor proteins of the viral capside; and a noncoding region called the long control region (LCR), which is located between L1 and E6 and contains the major part of regulatory elements involved in viral DNA replication and transcription.[53]

HPV has tropism for epithelial cells and depends on their differentiation to complete its own life cycle. The virus penetrates through microwounds of mucosa and skin, reaching first the basal cell layer of epithelium where it infects proliferating keratynocytes.[62] In this phase, although at very low rates, expression of products of early genes (E1, E2, E4, E5, E6 and E7) occurs, increasing infected cell proliferation and lateral expansion. Next, part of the progeny migrates to the suprabasal layers, where viral genes L1 and L2 are activated (late proteins). The viral genome is replicated in suprabasal cells, and whole viral particles are assembled and released.[19] The main strategy of HPV is to stay invisible to the host immune system, which cannot start primary immune reaction since the infection is restricted to the epithelium, and HPV has many evasion mechanisms. The interactions between these evasion mechanisms and the host immune system determine if the virus will be cleared or will stay as a persistent infection, which represents a major risk for malignant development.[62]

The virus follows the life cycle of epithelial cell from its formation in the basal layer, where infected cell expansion starts, passing through maturation in the upper layers and being released with cell apoptosis on the epithelial surface. However, this happens without cell lysis and, therefore, without the stimulation of immune system cytokines. Also, antigen presentation by Langerhans cells is weak[19,63,64] since HPV E7

protein reduces E-caderin expression, which is responsible for Langerhans cells adhesion to keratinocytes.[65,66] HPV can stay in epithelial cells either in the episomal form (low-risk subtype) or integrated to cell DNA (high-risk subtypes). High risk HPV blocks cell exit from the cell cycle, and S phase is maintained in the suprabasal differentiated cells by E6 and E7 proteins, which leads to cell immortality. Eventually, malignant transformation occurs with events such as cell architecture disorganization, suprabasal mitotic activity, malignant transformation of epithelial cells and consequent basal cell rupture and cell invasion into connective tissue, characterizing invasive carcinoma (Fig. 2).[19,63,64,67-70]

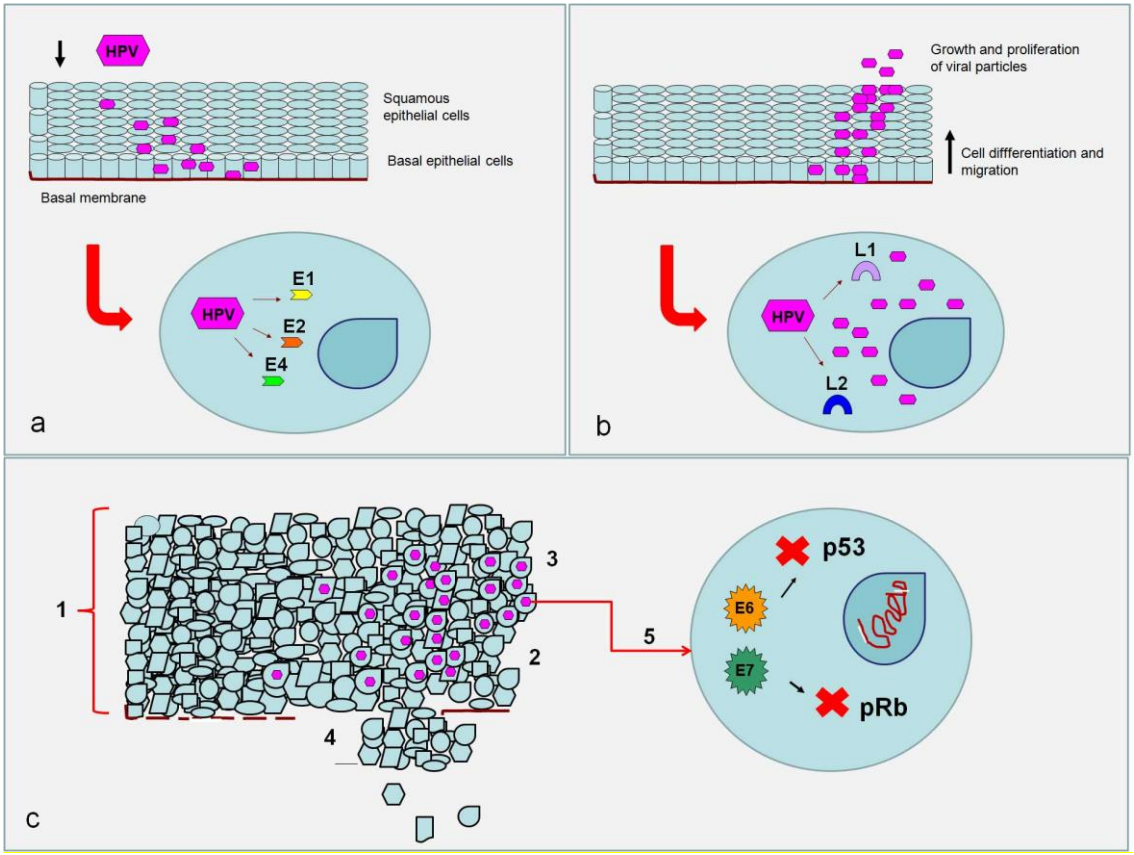

Figure 2 - Infection of epithelial cells by HPV and mechanisms of cell transformation. Normal epithelium is infected by HPV, and viral episomal replication occurs with expression of E1, E2 and E4 proteins (a); expression of L1 and L2 proteins with growth and proliferation of viral particles (b). Malignant transformation (c): disorganized cell architecture (1), suprabasal mitoses (2), epithelial cells transformed by HPV (3), rupture of basal membrane and cell invasion into connective tissue characterizing invasive carcinoma (4), integration with host chromosome and expression of E6 and E7 proteins blocking p53 and pRb oncosuppressive action (5)

Two products of the high-risk HPV genome are capable of forming specific complexes with cell cycle regulators: E6, which binds to p53 inducing its degradation; and E7, which blocks the oncosuppressive activity of retinoblastoma susceptibility protein (pRb). After viral integration, the expression of E6 and E7 oncoproteins triggers a series of malignant transformation processes, including cell cycle control deviation, synthesis of DNA, apoptosis inhibition and activation of the transcription of genes that promote cell proliferation.[19,53,66,71]

Disturbances involving p53 tumor suppressor gene (TP53) and its protein p53 occur in a large spectrum of human malignancies. p53 protein does not function properly in most of human cancers. Its inactivation happens as a result of (a) mutations in the TP53 gene, (b) alterations in genes whose products interact with or transmit information to or from p53, and (c) binding to viral proteins. The last is typical of lymphomas as well as cervix and liver tumors.[72] Although the role of p53 in tumor progression is still not clarified, its cumulative detection and the identification of the number and nature of TP53 mutations may have prognostic importance.[73] Losing p53 function is so detrimental, that it occurs in almost all human cancers.[72] In case of HPV, the protein coded by the E6 gene of HPV 16 and 18 binds to normal p53 increasing its degradation. This mechanism plays an important role in the pathogenesis of HPV-associated carcinomas.[74]

HPV and head and neck carcinoma

HPV has been detected in diverse head and neck tumors.[60,75-77] Data suggest that between 15 and 20% of squamous cell carcinomas in this region are associated with HPV infection,[76] especially HPV 16, with those in the oropharynx, tonsils and base of the tongue being the most affected intraoral sites.[60,77]

The role of HPV infection in oral cancer was first suggested by Syrjänen *et al.*[18] Since then, various studies have reported HPV prevalence in both oral tumors and precancerous lesions, varying from 0 to 100%, which reflects the variability inherent to the populations studied and different detection methods used.[11] The growing annual incidence of HPV infection in oropharyngeal carcinomas points to the estimate that by 2020 in the USA, the total number of oropharyngeal cancers associated with this virus will surpass uterine cervix cancers.[3]

Persistent HPV infections, especially high-risk types such as HPV 16,[77,78] are involved in the carcinogenesis of a particular group of head and neck squamous cell carcinomas[5,77,78] that differ from other tumors in clinical and histopathological aspects.[60] HPV-positive squamous cell carcinomas are more often localized in the oropharynx, and the incidence of carcinomas at this site has increased. These tumors have distinct and particular features, as well as unique demographic behavior and risk factors. Patients tend to be Caucasian,[5] younger than 40 years of age, and commonly non-smokers and non-alcohol drinkers, with a male:female prevalence ratio of 4:1[60] and higher cummulative sexual exposure and practices (oral sex, casual sex, number of partners), compared to HPV-negative squamous cell carcinoma patients. Actually, HPV-related head and neck cancer incidence (tonsils, base of tongue and oropharynx) is higher in young adults, in part because of changes in sexual behavior.[5]

On clinical examination, these squamous cell carcinomas appear as a small or occult primary tumor with advanced neck compromise. On microscopic examination, they are non-keratinized tumors with basaloid characteristics, excessive mitoses and comedonecrosis. Such tumors have a particular immunohistochemical profile characterized by strong and diffuse reactivity to p16, low or negative expression of p53 and high scores of Ki67.[60] There is some difficulty in the early diagnosis of these

carcinomas that grow in the palatine and lingual tonsils and palate, because initially, they are often asymptomatic and have no other evident clinical manifestation. Absence of detectable precancerous lesions of HPV-related oropharyngeal cancer makes visual inspection ineffective in the screening of these tumors. Because of their deep localization, neither clinical examination nor cytopathological analysis such as Papanicolaou is reliable for early detection. Such small hidden tumors are many times associated with neck metastases, whereas the most advanced ones can also determine dysphagia and otalgia, local pain and foreign body sensation in the throat.[60]

According to Paolini *et al.*,[78] cutaneous and mucosal types of HPVs can infect the oral epithelium, but only the mucosal ones, especially HPV-16, are clearly associated with tumors. Stokes *et al.*,[79] in turn, detected HPV in verrucous and papilomatous lesions by *in situ* hybridization, PCR, genotyping and immunohistochemistry and concluded that although DNA of high-risk HPV is detectable in a subgroup of verrucous malignant and dysplastic oral lesions, the lack of overexpression of p16 suggests that the oncogenic process is not determined by this factor. Anyway, considering their small sample size, the authors pointed out the need for further studies disclosing the biological meaning of the virus in such lesions.

Non-keratinized squamous cell carcinomas of the tonsils and base of the tongue showed high prevalence of HPV DNA.[77] However, the mere detection of viral DNA is insufficient evidence for cause-and-effect relationship. Moreover, the expression of HPV E6 and E7 oncogenes is necessary to determine tumor initiation and is therefore still the gold standard for classifying a malignant tumor as HPV-related.[71,80,81] Lingen *et al.*[82] analyzed the expression of HPV E6 and E7 oncogenes in 409 oral squamous cell carcinomas. HPV-positive tumors occurred in any region of oral cavity (floor of the mouth, anterior region of the tongue, alveolar process, hard palate, gingiva and lip) and

showed association with males, early stage, poor differentiation and basaloid histopathology.

Immunotherapy for HPV

HPV detection in head and neck squamous cell carcinomas is of extreme clinical importance, since patients with virus positivity can show a different clinical course compared to HPV-negative patients.[76] Also, additional therapeutic modalities including immunotherapy with vaccines could be used in such cases.

The efficacy of bivalent (16 and 18 types) and quadrivalent (6, 11, 16 and 18 types) vaccines against cervical and anal HPV has been showed.[83] The available vaccines (Gardasil® and Cervarix®) have been highly efficient against HPV-16 in genital lesions in both sexes.[83,84] Although recent data point to the hypothesis of efficacy of such vaccines also in preventing oral HPV infection,[83] they have not yet been administered for this purpose. Nonetheless, considering that HPV-16 is the most common subtype in the oral cavity as in the uterine cervix,[83,84] that seems to be a rational indication.

In a randomized double-blind clinical trial, Herrero *et al.*[83] evaluated the efficacy of a bivalent vaccine in reducing oral HPV infection. In the first phase of the study authors observed a 93.3% reduction in the prevalence of oral HPV 16/18 infection approximately four years after vaccination. The study did not provide direct and sufficient evidence that the vaccine prevented oropharyngeal carcinoma. Nevertheless, its high efficacy against oral HPV16/18 infection supports the idea that vaccination could reduce the risk for HPV-positive oropharyngeal carcinomas, especially those positive for HPV 16, the virus type most associated with this cancer, favoring primary prevention of such tumors.

Simultaneous infection with HPV and EBV in head and neck carcinomas

Mirzamani *et al.*[10] tested nasopharyngeal carcinoma samples for EBV and HPV by *in situ* hibridization. Two out of 20 samples (10%) had HPV 6/11 DNA sequence and 2 others (10%) had sequencies of HPV 16/18 DNA, whereas 19 samples (95%) showed EBV positivity. Combined infection of EBV and HPV was detected in 3 cases (15%). Higa *et al.*[40] found an unexpectedly high prevalence of EBV (72%) and HPV (78%) infection in well-differentiated oral squamous cell carcinomas. According to the authors, EBV and HPV16 are risk factors for squamous cell carcinomas of the tongue and pharyngolarynx, exerting oncogenic effects in these tumors. However, the specific role of coinfection by the two agents in carcinomas still needs to be determined.[2]

FINAL CONSIDERATIONS

Head and neck tumor investigations involve many different methods, where the role of high-risk HPV and EBV infection and molecular alterations underlying tumor progression are important targets (Table 1). A major role has been attributed to these viruses in oral malignancies, especially nasopharyngeal carcinomas.[2] On the other hand, HPV has not been detected in salivary gland tumors, suggesting it does not play any role in the etiopathogenesis of these tumors. Also, EBV does not seem to play a major role in the development of such lesions.[85]

Although effective vaccines are already available for preventing with hepatitis B virus and HPV subtypes respectively associated with hepatocellular carcioma and uterine cervix carcinoma, a vaccine for EBV is still unavailable.[48] Besides, even though already administered to prevent genital HPV, vaccines have not yet been considered for oral infection. Nonetheless, taking into account that HPV 16 is a risk factor for uterine

cervix and oropharyngeal carcinomas, it would be reasonable to use the vaccine also to reduce oral HPV infection rates.[84] Moreover, epidemiological data show that the prevention of EBV-associated diseases by means of vaccination would have a great impact on public health and related health care costs.[48] Availability of vaccines against high risk HPV 16 and 18, and EBV vaccine, with the latter still in phase II clinical trials, could provide preventive measures against malignancies associated with these viruses.[2,48-50]

Head and neck carcinogenesis related to EBV and HPV requires new investigations with a large number of observations and standardized methodology allowing a better understanding of these processes. Up to now, published results are controversial and vary according to geographic distribution of the study population and methods employed.[2,8,86] Anyway, the growing spectrum of knowledge in tumor virology gives us positive perspectives, and further research investigating the multiple mechanisms used by viral agents in tumor progression is necessary to develop new preventative and therapeutic strategies.[7]

Table 1: Reports on association of *Human papillomavirus* and *Epstein-Barr virus* with head and neck tumors

Neoplasm	Method	Virus (marker)	Detection rate n/total (%)	Reference
Oral SCC	Immunoperoxidase	HPV	16/40 (40%)	Syrjänen *et al.*[18]
Oral SCC	nPCR	EBV (DNA)	OSCC: 11/29 (37.9%) Controls: 5/67 (7.3%)	Sand *et al.*[41]
Oral SCC	PCR	EBV (LMP1; EBNA2) HPV (E6 e E7)	128 /177(72%) 139 /177 (78%)	Higa *et al.*[40]
Oral SCC	IHC	EBV (LMP1)	48/65 (73.8%)	Kis *et al.*[14]
Oral SCC	PCR/DNA	HPV EBV	54 /217 (24.9 %) 69/217 (31.8 %)	Jalouli *et al.*[29]
Oral SCC	PCR IHC	HPV (E6/7) HPV (p16) Other HPVs	24/409 (5.9%) 15/409 (3.7%) 9/409 (2.2%)	Lingen *et al.*[82]
Oropharyngeal SCC	ISH Inno-LiPA Viral load mRNA	HPV16 Any HPV HPV 16 HPV16 E6/E7	76/271 (28,0%) 116/263 (44.1%) 81/263 (30.8%) 76/216 (35.2%)	Chaturvedi *et al.*[3]
Oropharyngeal carcinoma	PCR	HPV (DNA/ E7)	Mucosal types: 17/78 (21.8%) Cutaneous types: 16/78 (20.5%)	Paolini *et al.*[78]
Nasopharyngeal carcinoma	Immunoblotting	EBNA 1 EBNA2 EBNA3 EBNA-LP LMP	24/24 (100%) 0/24 (0%) 0/24 (0%) 0/24 (0%) 9/24 (37.5%)	Young *et al.*[87]
Nasopharyngeal carcinoma	PCR	EBV HPV EBV/ HPV	20/63 (32%) 12/63 (19%) Co-infection not found	Giannoudis *et al.*[75]
Nasopharyngeal carcinoma	ISH IHC PCR	EBV (EBER) EBV (LMP1) EBNA1, LMP1, LMP2 EBNA2	120/120 (100%) 36/50 (72%) 4/4 (100%) 0/4 (0%)	Pathmanathan *et al.*[38]

Neoplasm	Method	Virus (marker)	Detection rate n/total (%)	Reference
Nasopharyngeal carcinoma	ISH	EBV (EBER) HPV 16/18 HPV 6/11 EBV & HPV	19/20 (95%) 2/20 (10 %) 2/20 (10 %) 3/20 (15%)	Mirzamani *et al.*[10]
Nasopharyngeal carcinoma	PCR (serum)	EBV (EBNA2) EBV (LMP1)	75/75 (100.0%) 44/75 (58.7%)	Tiwawech *et al.*[43]
HNSCC	PCR, ISH	HPV16 (L1) HPV16 (E7)	L1: serum: 0/67; tumor: 15/51 (29%) E7: serum: 4/66 (6%); tumor: 15/70 (21%)	Capone *et al.*[76]
HNSCC (neck metastasis)	ISH	HPV	10/30 (33%)	Zhang *et al.*[88]
SCC (oropharynx; oral cavity)	PCR q-PCR	HPV16	SCC oropharynx: 12/12 (100%) SCC oral cavity: 0/12 (0%)	Laborde *et al.*[21]
SCC (tongue, pharyngeal, laryngeal)	PCR	EBV HPV18 HPV 16	99/122 (81.15%) 15/122 (12.3%) *highest number of copies*	Zheng *et al.*[20]
Carcinoma of tonsil and base of tongue	PCR, Inno-LiPA	HPV 16 HPV 31 HPV 33	10/20 (60%) 1/20 (5%) 1/20 (5%)	El-Mofty; Patil[77]
	IHC	HPV (p16)	20/20 (100%)	
Verrucous carcinoma	PCR ISH IHC	HPV (16 genotype) HPV (low/high risk) HPV(p16)	1/7 (14.3%) 0/7 (0%) 0/7 (0%)	Stokes *et al.*[79]
DLBCL	PCR (blood/ bone marrow)	EBV genome	28/130 (21.5%)	Marques *et al.*[36]
Salivary gland neoplasms (adenocarcioma, lymphoma, pleomorphic adenoma)	PCR ISH	EBV HPV	2/38 (5.26%) (1 lymphoma; 1 pleomorphic adenoma) 0/38 (0%)	Atula *et al.*[85]

SCC*:* squamous cell carcinoma ; PCR: polymerase chain reaction; IHC: immunohistochemistry; HPV*:* human papilloma virus; ISH*: in situ* hybridization; EBV*:* Epstein -Barr virus; CMV*:* cytomegalovirus; NPC*:* nasopharyngeal carcinoma; HNSCC*:* head and neck squamous cell carcinoma; DLBCL: diffuse large B-cell lymphoma; *n*-PCR: nested polymerase chain reaction; *q*-PCR: quantitative polymerase chain reaction; E6*:* early gene 6 (oncoprotein of HPV); E7: early gene 7 (oncoprotein of HPV); LMP: latent membrane protein; EBER: Epstein–Barr virus encoded small RNAs; EBNA: Epstein–Barr virus nuclear antigen

HIGHLIGHTS

-Viruses interfere with host cell control mechanisms of growth and differentiation.

-A major role has been attributed to HPV and EBV in head and neck malignancies.

-Head and neck carcinogenesis related to EBV and HPV requires further studies.

ACKNOWLEDGMENTS

We thank Dr. A. Leyva (U.S.A) for English editing of the manuscript.

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3 ARTIGO 2

O artigo a seguir intitula-se ***Relationships of Epstein-Barr virus (EBV), Human papillomavirus (HPV), Ki-67, p53 and clinical features in oral squamous cell carcinoma and oral lymphoma*** e foi formatado de acordo com as normas e submetido ao periódico *Head & Neck* (Anexos C e D).

Relationships of *Epstein-Barr virus* (EBV), *Human papillomavirus* (HPV), Ki-67, p53 and clinical features in oral squamous cell carcinoma and oral lymphoma

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Acknowledgments

We thank Dr A. Leyva (U.S.A.) for English editing of the manuscript.

Running title: *EBV and HPV in oral tumors*

Key words: squamous cell carcinoma; lymphoma; viruses; EBV; HPV

ABSTRACT

Background: Human papillomavirus (HPV), *Epstein-Barr virus* (EBV) and clinical and histological features were investigated in oral cancer.

Methods: Clinical features and HPV, EBV, p53 and Ki-67 immunostaining were analyzed in oral squamous cell carcinoma (OSCC), lymphoma and fibrous hyperplasia.

Results: HPV and EBV were not associated with the tumors analyzed. p53 expression was significantly lower in lymphoma and fibrous hyperplasia than OSCCs. OSCC grade II and III were associated with male gender and smoking, while grade III was also associated with alcohol. Duration and size of the lesion were inversely correlated; lesion size was also inversely correlated to HPV. EBV and HPV were positively correlated to each other, as was Ki-67 to lesion size and p53, which was correlated to HPV.

Conclusion: The results do not support an association of HPV and EBV with OSCC and oral lymphoma. Positive correlation between HPV and EBV suggested co-infection.

INTRODUCTION

Oral cancer is a multifactorial disease associated with intrinsic and extrinsic factors, where tobacco and alcohol play a major role.[1-5] Nevertheless, more recently, the participation of viruses in oral cancer development has been discussed, and it has been pointed out that *Epstein-Barr virus* (EBV) and *Human papillomavirus* (HPV) are the viral agents most associated with oral tumors.[3,5-7] Also, co-infection with two or more viruses has been suggested as a factor that increases the risk for cancer development, including head and neck squamous cell carcinoma.[8,9]

EBV has been consistently associated with lymphoma and nasopharyngeal carcinoma,[6,10,11] but its association with oral squamous cell carcinoma (OSCC) has not yet been definitely proved.[12-15] Regarding HPV, up to now, more than 170 genotypes[16,17] infecting the mucosa of the oral and genital tracts have been identified and classified according to oncogenic potential: low-risk subtypes (e.g., types 6 and 11), which are mainly associated with benign genital warts; and high-risk subtypes (e.g., 16 and 18), which are etiological agents of uterine cervix carcinoma.[18]

Human oncogenic viruses are defined as necessary but not sufficient to initiate cancer.[19,20] Experimental evidence suggests that the oncogenic potential of a virus is effective in cells that have already accumulated a certain number of genetic mutations leading them to cell cycle deregulation. The current model of viral oncogenesis is not capable of explaining the development of tumors in tumorigenic virus carriers, which is considered a rare event that occurs some decades after infection.[20] According to some reports, EBV can accelerate host cell malignant transformation.[21] This occurs mainly because of latent membrane protein 1 (LMP1), which is frequently expressed in EBV-associated cancer and considered its most potent oncogenic protein. It has been observed *in vitro* that LMP1 promotes cell growth, increases cell motility,

protects cells from apoptosis and promotes angiogenesis.[22,23] Moreover, two products from high-risk HPV genome are capable of forming specific complexes with cell cycle regulators. E6 binds to p53 inducing its degradation, and E7 interacts with pRb (retinoblastoma susceptibility protein) blocking its activity. After viral integration, the expression of E6/E7 oncoproteins triggers a series of malignant transformation processes, including disruption of cell cycle control and DNA synthesis, inhibition of apoptosis and activation of transcription of genes that promote cell proliferation.[24]

HPV has been related to potentially malignant oral lesions as well as to head and neck squamous cell carcinomas, including oral and mainly oropharyngeal[25-28] and base of the tongue ones.[28] Clinical, epidemiological and molecular evidence has shown that especially HPV type 16 is involved in such lesions.[28] Also, Higa *et al*.[29] found a high prevalence of EBV (72%) and HPV (78%) infection in OSCCs. On the basis of these reports, EBV and HPV16 have been considered risk factors for squamous cell carcinomas of the tongue and pharyngolarynx. Anyway, there are still many disagreements regarding this issue,[12-15] which demands further investigations. The aim of this study was to investigate HPV, EBV, cell proliferation rate (Ki-67) and p53 in OSCC and oral lymphoma, considering clinical and histological features of the tumors.

MATERIAL AND METHODS

This study was approved by the Ethics Research Committee of the Pontifical Catholic University of Rio Grande do Sul. The sample was composed of archived material (medical records and paraffin blocks of biopsied specimens) from patients with oral squamous cell carcinoma (OSCC), oral lymphoma and oral traumatic fibrous hyperplasia. The biopsied specimens and medical records were allocated into 5 groups: (1) 16 samples from patients with OSCC grade I (well-differentiated); (2) 16

samples from patients with OSCC grade II (moderately-differentiated); (3) 19 samples from patients with OSCC grade III (poorly-differentiated); (4) 14 samples from patients with oral non-Hodgkin lymphoma; and (5) 19 samples from patients with oral traumatic fibrous hyperplasia. OSCC histological grading followed the WHO criteria.[30] All lesions were intraoral, affecting different sites: tongue, soft palate, hard palate, floor of the mouth, buccal mucosa and alveolar ridge (maxilla). Lesions of vermilion border of the lip were excluded. The lymphoma group included Burkitt's lymphoma (n=1), diffuse large B-cell lymphoma (n=6), non-Hodgkin lymphoma without other specification (n=7).

The inclusion criteria were: records properly registered and paraffin blocks in adequate conditions for analysis. The following data were collected from the records: (1) age and gender of the patient; (2) duration and size of the lesion at diagnosis; (3) habits (alcohol, tobacco and *chimarrão* use); and (4) comorbidities [HIV infection or other immunological disturbances; proliferative verrucous leukoplakia (PVL), lichen planus; previous history of cancer].

Histological processing

The specimens were processed by hematoxylin and eosin staining (H&E) according to standard techniques. The H&E slides were used to confirm the diagnosis and histological classification of the lesions. Next, the specimens were processed for immunohistochemistry.

Immunohistochemistry (IHC)

Three-micrometer-thick tissue sections were obtained using Leica RT2125 microtome (Leica, Melbourne, VIC, Australia) and mounted on positive-charged slides (Super Frost, Thermo Scientific, Loughborough, LC, UK). Antigen retrieval was done with

heat (HIER) in pTLink (Dako, Glostrup, Denmark) for 30 min at 98°C, and the slides were washed with phosphate-buffered saline (PBS, pH 7.2). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 15 min. Slides were incubated for 1 h by the capillarity method in a Sequenza station (Thermo Shandon, Waltham, MA, USA) at room temperature (24ºC) with Flex anti-Ki-67 antigen (clone MIB-1, Dako), Flex anti-p53 protein (clone DO-7, Dako), Flex anti-EBV-LMP (clone CS-1.4, Dako), and anti-HPV broad spectrum (Biocare Medical, Rio de Janeiro, RJ, Brazil) 1:50 in antibody diluent with background reducing components (Dako). Advanced HRP Kit (Dako) was used for signal amplification, and staining revelation was with diaminobenzidine chromogen (DAB, Dako). Counterstaining was performed with Harris hematoxylin, and the slides were cleared with xylene and coverslipped with Dako coverslipper. Histological specimens of HPV-positive cervical carcinoma, colon carcinoma, EBV-positive Burkitt's lymphoma, and tonsil served as positive controls for HPV, p53, EBV (LMP1) and Ki-67, respectively. Omission of the primary antibody was used as negative control.

Histological analysis

Histological images were digitized by means of a Zeiss Axioskop 40 light microscope (Zeiss, Oberkohen, Germany), connected to a QImaging Retiga-2000R videocamera (QImaging, Surrey, BC, Canada) and a computer with Image Pro Capture Kit (Media Cybernetics, Silver Spring, USA). Images were captured by using a 20x objective (Ki67 and p53) and a 40x objective [HPV and EBV (LMP1)] and stored as non-compressed TIFF (True Image Format File). Ten fields were captured per slide in a standardized manner.

The analysis was performed by one blind calibrated observer in Image Pro Plus 4.5.1 software (Media Cybernetics). IHC images were evaluated by means of a semi-

automated segmentation technique,[31] where the strong brown stained area (μm^2) was quantified. Calibration consisted of evaluating a series of 20 images at two different times. Agreement between the results of these two evaluations was tested by intraclass correlation coefficient, which resulted in $r>0.7$.

Statistical analysis

Data were analyzed by means of descriptive (frequency, mean, median, standard-deviation, 25th percentile, 75th percentile) and inferential statistics. Shapiro-Wilk was used to test for normal sample distribution. Immunohistochemical expression of EBV, HPV, p53 and Ki-67 was compared between the groups by using Kruskal-Wallis complemented by the Student-Newman-Keuls test. Clinical variables were analyzed with ANOVA and the chi-square test complemented by adjusted residual analysis, and correlations were tested by the Spearman correlation coefficient. Statistical analysis was performed in SPSS 18.0 (Statistical Package for the Social Sciences (IBM, Armonk, NY, USA) setting the level of significance at 5%.

RESULTS

IHC analysis for HPV, EBV, Ki-67 and p53 (Fig.1)

HPV immunostaining was significantly lower in the lymphoma group compared to the other groups, but it did not differ between carcinomas and fibrous hyperplasia. EBV staining, in turn, did not significantly differ between the groups analyzed (Table 1, Kruskal-Wallis, Student-Newman-Keuls, $\alpha=0.05$). Ki-67 staining was significantly lower in the fibrous hyperplasia group compared to the other groups, and it did not show any significant differences between lymphoma and carcinomas. The lymphoma and fibrous hyperplasia groups showed significantly lower p53 staining than the

carcinoma groups, with no other significant differences for this variable (Table 2, Kruskal-Wallis, Student-Newman-Keuls, α=0.05).

Table 1 – Immunostaining (μm^2) for HPV and EBV in the oral squamous cell carcinoma (OSCC) grade I, II and III, lymphoma and fibrous hyperplasia groups

	HPV			EBV		
Group	**MD**	**P25**	**P75**	**MD**	**P25**	**P75**
OSCC I	588.21^{A}	211.63	905.48	1.85 ^{A}	0.000	173.69
OSCC II	811.09^{A}	508.70	1077.59	371.72 ^{A}	98.12	607.66
OSCC III	805.06^{A}	241.85	964.47	32.40 ^{A}	0.55	353.10
Lymphoma	**81.22^{B}**	0.000	182.37	51.03 ^{A}	2.51	155.78
Fibrous hyperplasia	978.83^{A}	333.63	1449.07	162.12 ^{A}	34.36	504.25

HPV=*Human papillomavirus*; EBV=*Epstein-Barr virus*; MD=median; P25=25th percentile; P75=75th percentile
Medians followed by different letters in a column differ significantly, Kruskal-Wallis, Student-Newman-Keuls, α=0.05

Table 2 – Immunostaining (μm^2) for Ki-67 and p53 in the oral squamous cell carcinoma (OSCC) grade I, II and III, lymphoma and fibrous hyperplasia groups

	Ki -67			p 53		
Group	**MD**	**P25**	**P75**	**MD**	**P25**	**P75**
OSCC I	1472.69^{A}	298.68	2757.32	1780.57^{A}	480.69	3916.56
OSCC II	2060.95^{A}	953.64	2747.25	1030.36 ^{A}	168.53	4589.39
OSCC III	1903.70^{A}	928.90	2809.11	2137.29 ^{A}	358.38	5754.18
Lymphoma	1359.46^{A}	458.29	2258.53	**74.81 ^{B}**	9.20	373.00
Fibrous hyperplasia	**494.02^{B}**	178.33	832.78	**240.01 ^{B}**	134.24	372.41

MD=median; P25=25th percentile; P75=75th percentile
Medians followed by different letters in a column differ significantly, Kruskal-Wallis, Student-Newman-Keuls, α=0.05

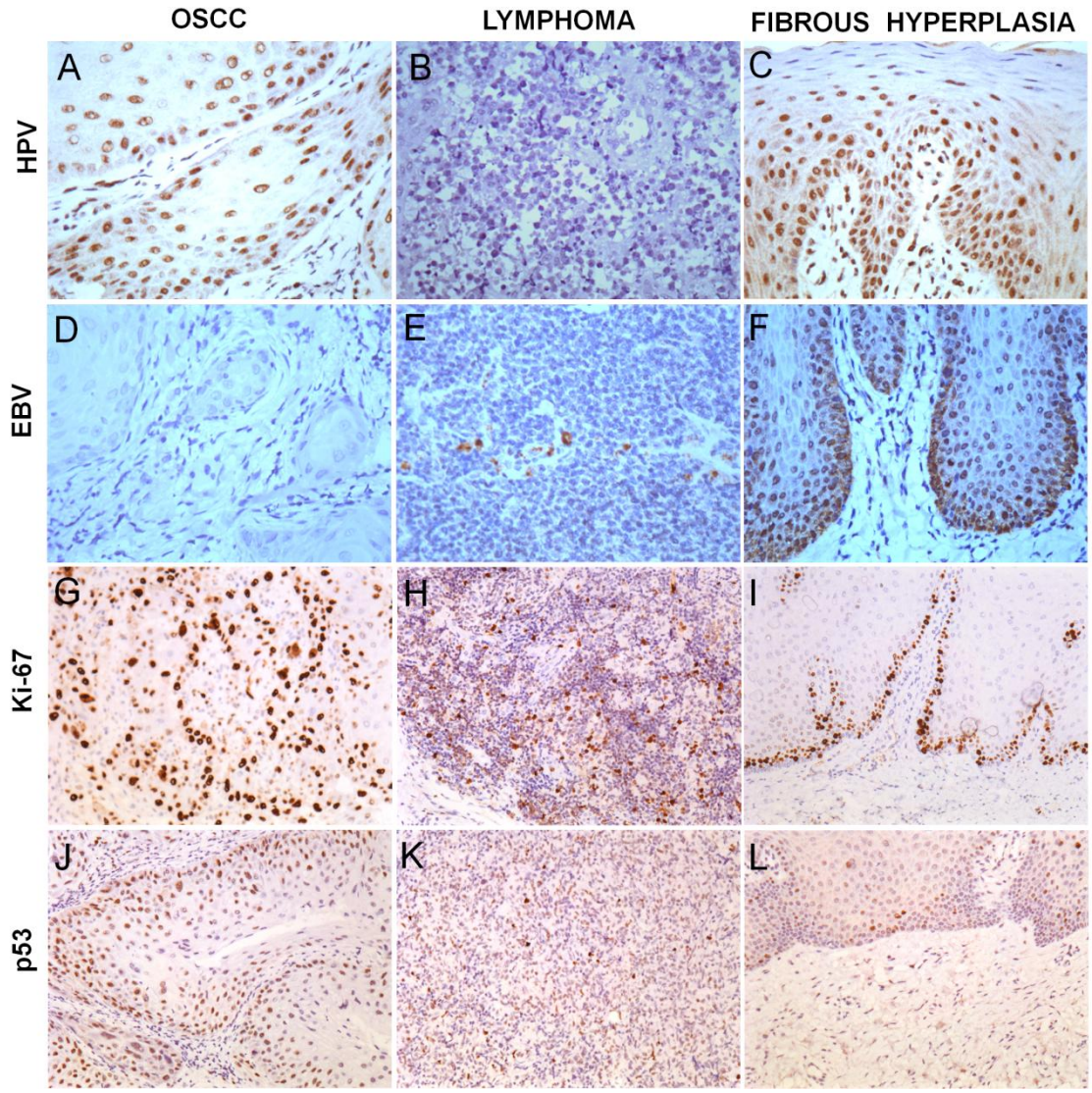

Figure 1 – HPV immunostaining (400x): (A) HPV-positive oral squamous cell carcinoma (OSCC) grade II; (B) HPV-negative Burkitt's lymphoma; (C) HPV-positive fibrous hyperplasia. **EBV immunostaining (400x):** (D) EBV-negative OSCC grade I; (E) EBV-positive diffuse large B-cell lymphoma (DLBCL); (F) EBV-positive fibrous hyperplasia. **Ki-67 immunostaining (200x):** (G) OSCC grade II; (H) lymphoma (DLBCL); (I) fibrous hyperplasia. **p53 immunostaining (200x):** (J) OSCC grade II; (K) lymphoma (DLBCL); (L) fibrous hyperplasia

Clinical features

Age and gender of the patients

OSCC grade II and III groups showed an association with male gender (**Fig.2**, chi-square, adjusted residual analysis, α=0.05), and there was no significant difference in age of the patients between the groups (**Fig.3**, ANOVA, P>0.05).

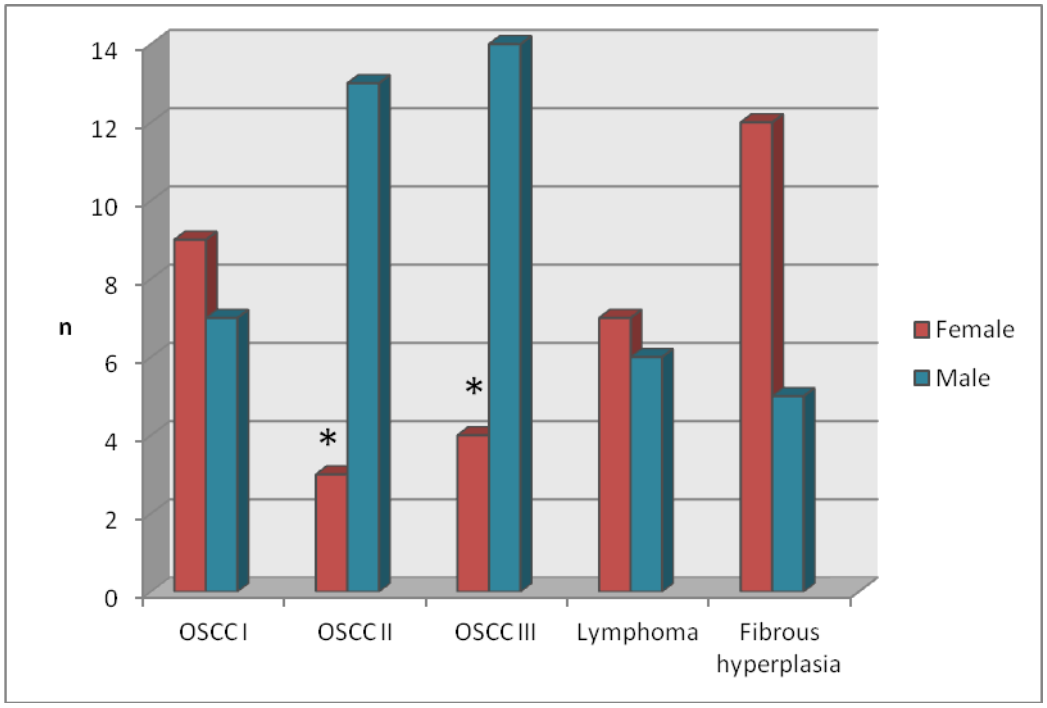

Figure 2 - Distribution of the patients according to gender in the oral squamous cell carcinomas (OSCC) grade I, II and III, lymphoma and fibrous hyperplasia groups.
*Chi-square test, adjusted residual analysis, $P<0.05$

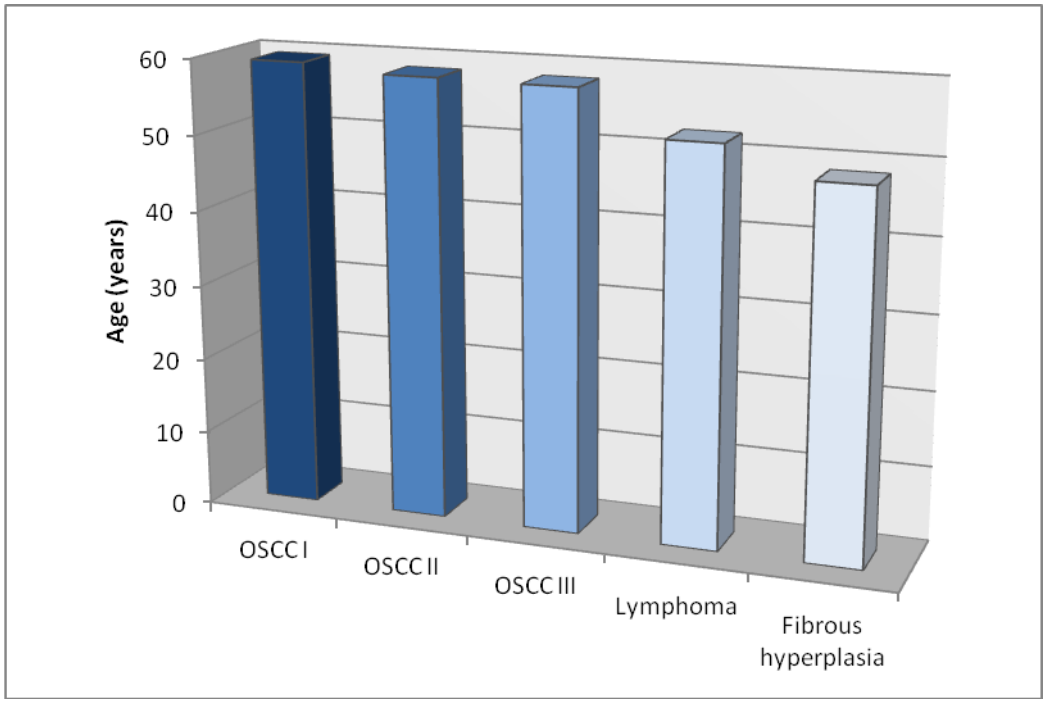

Figure 3 - Mean age of the patients in the oral squamous cell carcinomas (OSCC) grade I, II and III, lymphoma and fibrous hyperplasia groups. ANOVA, $P>0.05$

Habits and comorbidities

The OSCC grade II and III groups were associated with tobacco smoking, whereas OSCC grade III was also associated with alcohol consumption. *Chimarrão* and comorbidities did not show any association with the groups (Table 3, chi-square test, adjusted residual analysis, α=0.05).

Table 3 – Distribution of patients according to habits and comorbidities in the oral squamous cell carcinoma (OSCC) grade I, II and III, lymphoma and fibrous hyperplasia groups

Group	Tobacco				Alcohol				*Chimarrão*				Comorbidities			
	Present		Absent		Present		Absent		Present		Absent		Present		Absent	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
OSCC I	8	50	8	50	3	18.8	13	81.3	3	18.8	13	81.3	7	13.8	9	56.3
OSCC II	**13***	**81.3**	3	18.8	7	43.8	9	56.3	5	31.3	11	68.8	4	25	12	75
OSCC III	**15***	**78.9**	4	21.1	**11***	**57.9**	8	42.1	1	5.3	18	94.7	2	10.5	17	89.5
Lymphoma	3	21.4	11	78.6	0	0	14	100	3	21.4	11	78.6	6	42.9	8	57.1
Fibrous hyperplasia	2	10.5	17	89.5	0	0	19	100	5	26.3	14	73.7	6	31.6	13	68.4

*Chi-square test, adjusted residual analysis, α= 0.05

Duration and size of the lesions

The duration of the lesion was significantly longer in fibrous hyperplasia group than the OSCC grade III and lymphoma groups; no other significant differences were observed for this variable between the groups. The lesion was significantly smaller in fibrous hyperplasia compared to the other groups, and also significantly smaller in OSCC grade I compared to OSCC grade III and compared to lymphoma. No other significant differences were observed for this variable (Table 4, Kruskal-Wallis, Student-Newman-Keuls, α=0.05).

Table 4 – Duration and size of the lesions in the oral squamous cell carcinoma (OSCC) grade I, II and III, lymphoma and fibrous hyperplasia groups

Group	Duration (months)			Size (cm)		
	Mean	SD	Median	Mean	SD	Median
OSCC I	13.68	15.98	5.00[AB]	2.62	3.11	1.50 [A]
OSCC II	7.44	14.27	4.00 [AB]	4.31	3.68	2.75 [AB]
OSCC III	2.94	1.74	2.00 [A]	4.83	3.07	4.50[B]
Lymphoma	12.03	34.05	2.00 [A]	5.39	4.17	3.00[B]
Fibrous hyperplasia	24.96	33.51	9.00 [B]	0.58	0.22	0.50[C]

SD=standard deviation
Medians followed by different letters in a column showed significant difference, Kruskal-Wallis, Student-Newman-Keuls, α=0.05

Correlations

In the general analysis, duration of the lesion was inversely correlated to lesion size, which in turn was inversely correlated to HPV. EBV and HPV were positively correlated to each other as was Ki-67 to lesion size and p53, which was correlated to HPV. Analysis within groups showed negative correlation between Ki-67 and duration of the lesion in OSCC grade I as well as between Ki-67 and HPV in the lymphoma group. No other correlations were observed (Table 5, Spearman's correlation coefficient, α=0.05).

Table 5 - "*r*" value for general and within group correlations according to Spearman's correlation coefficient

General	Age	Lesion size	Duration	HPV	EBV	Ki-67	p53
Age	1						
Lesion size	0.016	1					
Duration	0.162	**-0.307****	1				
HPV	0.019	**-0.267***	0.188	1			
EBV	0.005	-0.144	0.154	**0.305****	1		
Ki-67	0.059	**0.366****	-0.206	0.137	0.044	1	
p53	0.017	-0.034	-0.037	**0.306****	-0.059	**0.356****	1
OSCC I							
Age	1						
Lesion size	-0.156	1					
Duration	0.185	-0.478	1				
HPV	-0.178	-0.389	-0.064	1			
EBV	0.086	-0.389	0.064	0.396	1		
Ki-67	-0.213	0.076	**-0.542***	0.494	0.350	1	
p53	-0.068	0.042	-0.226	0.361	-0.017	0.182	1
OSCC II							
Age	1						
Lesion size	0.077	1					
Duration	0.100	0.159	1				
HPV	0.099	0.065	-0.024	1			
EBV	0.283	-0.213	-0.115	-0.077	1		
Ki-67	0.306	0.231	-0.090	-0.124	0.040	1	
p53	0.116	-0.317	-0.052	0.124	-0.203	0.324	1
OSCC III							
Age	1						
Lesion size	-0.043	1					
Duration	0.052	0.134	1				
HPV	0.114	0.205	0.290	1			
EBV	0.274	-0.145	0.339	0.296	1		
Ki-67	-0.006	0.019	0.375	0.158	-0.184	1	
p53	-0.120	-0.362	0.191	0.225	-0.025	0.149	1
Lymphoma							
Age	1						
Lesion size	-0.541	1					
Duration	0.497	-0.264	1				
HPV	0.338	-0.519	0.097	1			
EBV	0.036	0.000	0.227	0.247	1		
Ki-67	-0.159	-0.239	-0.414	**0.708****	0.196	1	
p53	-0.422	0.409	-0.093	0.070	-0.305	0.021	1
Fibrous hyperplasia							
Age	1						
Lesion size	0.201	1					
Duration	0.083	0.450	1				
HPV	-0.054	-0.092	-0.162	1			
EBV	-0.316	-0.166	0.018	0.065	1		
Ki-67	-0.045	0.014	0.449	0.090	0.127	1	
p53	0.134	-0.248	-0.263	0.306	0.053	-0.299	1

*Correlation at 0.05 level of significance; **Correlation at 0.01 level of significance.
OSCC=oral squamous cell carcinoma; EBV=*Epstein-Barr virus*; HPV=*Human papillomavirus*

DISCUSSION

Analysis of viral protein expression showed significantly lower HPV immunostaining in the lymphoma group with no other significant differences between the groups analyzed, even for EBV. Such result showing the rates of both viruses in the (traumatic) fibrous hyperplasia group not differing significantly compared to the tumor groups suggests a lack of association of either HPV or EBV with the oral malignancies evaluated, an idea already defended by other authors.[12-15] Also, it corroborates the occurrence of HPV and EBV in oral mucosa, regardless of causing oral lesions,[32] where the oral cavity could play a role as a reservoir for such viruses.[33,34]

Regarding HPV, one point to recall is that we used a broad spectrum antibody (HPV 1,6,11,16,18, and 31), which was therefore not restricted to detecting the high-risk HPVs but low-risk types as well, which might have contributed to the expression rates seen in the fibrous hyperplasia group. Moreover, according to some authors,[35] using just a single virus detection method may not be efficacious in confirming oropharyngeal carcinoma association with HPV. These factors could have led to biases in our study. On the other hand, the literature also reports high rates of HPV detection (86.3%) in oropharyngeal carcinomas by using IHC.[36] Anyway, our sample was composed of oral carcinomas but not oropharyngeal ones. Unlike pharyngeal carcinomas,[27,37-41] oral ones can show high rates of no association with HPV.[42-45] Stokes *et al.*,[46] in turn, by using IHC, *in situ* hybridization (ISH), consensus PCR and genotype analysis in oral lesions, found a lack of p16 overexpression even though high-risk HPV-16 DNA was highly detected in dysplastic and malignant verrucous lesions, and suggested that the oncogenic process may not be triggered by HPV. Accordingly, previous studies have shown a significant relationship between viruses and oropharyngeal carcinomas[37,47-51] but none has proved a direct association of HPV with OSCC development.[44,50,52-54]

EBV expression (LMP-1) did not significantly differ between the groups suggesting no association of EBV with either OSCC or oral lymphoma. Our result for OSCC disagrees with studies reporting this association.[55] Anyway, in agreement with our findings, Saravani *et al.*[56] do not support the hypothesis that EBV would be directly involved in OSCC development. Actually, EBV has been highly associated with undifferentiated nasopharyngeal carcinoma; however, its relationship with other head and neck neoplasms is still under debate.[57] Still, regardless of the lack of statistical significance, the lower expression of EBV in the OSCC grade I agrees with the results reported in the literature, according to which moderately differentiated carcinomas of the tongue and pharyngolarynx show higher expression of EBV than do well-differentiated ones.[40] On the other hand, considering the lack of association between EBV and lymphomas we found, it is important to emphasize that our group of these tumors was not restricted to Burkitt's lymphoma, which is the neoplasm most associated with EBV. Still, we have to recall that within Burkitt's lymphomas, the endemic type shows high association with EBV, with 90% prevalence, whereas rates range from 10 to 20% in the other types.[15] Moreover, even though LMP1 is expressed in most EBV-related human cancers,[58] sometimes it may not be expressed in the tumor,[32] including Burkitt's lymphoma,[59] in spite of the association of that tumor with EBV infection.[32,59] Another point to consider is that despite lack of statistical significance, EBV expression was higher in the fibrous hyperplasia group than in the lymphoma group. An intriguing finding that would support EBV occurrence not only in EBV-related oral lesions but in normal oral mucosa as well. Accordingly, Kis *et al.*[12] found that even patients with EBV-negative tumors had a notably high carriage rate of EBV in the healthy mucosa. In fact, the virus is considered ubiquitous,[60] occurring as a latent infection[61] with reports of seroprevalence of 95% in the world-wide population.[60]

Ki-67 expression was significantly lower in the fibrous hyperplasia group, and p53 expression lower in both the fibrous hyperplasia and the lymphoma groups, without any other significant differences, which was in agreement with the aggressive behavior of the malignancies compared to benign lesions and with the more determinant involvement of p53 overexpression in carcinoma pathogenesis than in lymphoma's.[62-64]

Concerning methodological aspects, the reports of high positivity rates for EBV and HPV[12,32-34,60,61] in normal oral mucosa led us to choose a quantitative method[31] in IHC analysis, expressing the rates of immunostaining and also performing statistical correlations. On the other hand, because of the ethical concerns involving the biopsy of healthy oral mucosa, oral traumatic fibrous hyperplasias were used as a control group.

Our results showed that tobacco and alcohol were associated with the most aggressive carcinoma groups (OSCC grade II and III), which corroborated the important role of these extrinsic factors in the etiopathogenesis of OSCC[14,65-68] regardless of association with HPV, *chimarrão* or comorbidities. Also, OSCC grade II and III were associated with male gender, which again corroborates the reports in the literature.[69,70] Age of the patients did not show any significant differences between the groups. According to the literature, OSCC associated with HPV has a distinct clinical behavior, affecting younger patients[33,71] and showing no strict relationship with tobacco and alcohol.[71] Such clinical behavior was not observed in our study, which anyway is in accordance with our finding of no significant differences for HPV. Maybe if we had used a sample composed of younger patients, these results could have been different.

The duration of the disease did not differ significantly between the malignancies; however, in the fibrous hyperplasia group it was significantly higher than in OSCC grade III and lymphomas. Here, it is also important to consider the subjectivity of this information, since it is an estimate given to us by the patient. The fibrous hyperplasia

group showed a significantly smaller lesion compared to the other groups, whereas OSCC grade I did not significantly differ from grade II, which, in turn, did not differ from grade III and lymphomas. However, the latter two groups showed significantly greater lesions than in OSCC grade I. Such findings suggest that OSCC grade III and lymphomas were the most aggressive tumors in our sample.

The inverse correlation between size and duration of the lesions seems to be explained by the malignant nature of the majority of them, which is corroborated by the positive correlation between Ki-67 and the size of the lesion. Moreover, the inverse correlation found between size of the lesion and HPV is in agreement with literature reports, according to which HPV-positive disease tends to present with smaller primary tumors but more advanced nodal stage.[40,41] The correlation between HPV and EBV, on the other hand, suggests the possibility of co-infection.[72] The correlation analysis within groups might have been impaired by the small size of the sample for this kind of evaluation.

The multifactorial nature of oral cancer makes it difficult to isolate one associated factor, and the complexity of the disease pathogenesis appears evident in the conflicting results of the various reported studies focusing on its relationship with HPV and EBV.[3,12,14,29,73-76] Besides, different detection methods in different populations with different location of the cancer lesion can contribute to divergence in results.[14] Some limitations of the IHC, such as false-positive results including cross-reactivity[77] and lack of consensus about interpretation criteria, might have also interfered with our results and with other reported findings as well. According to Fonmarty *et al.*,[35] it appears essential for future clinical trials to be stratified according to tumor HPV status, defined by means of reliable virological tests targeting E6/E7 mRNA, which demands the development of new tests suitable for use in routine practice. Eventually, further prospective studies using

very rigorous methodology, including standardization of techniques with high accuracy and large sample size rigorously stratified by age, anatomical site of the lesion and histological type of the tumor, are needed to define the association of HPV and EBV with oral cancer.

CONCLUSION

The results of the present study do not support an association of HPV and EBV with OSCC and oral lymphomas. Tobacco smoking and alcohol consumption were associated with OSCC grade II and III, but not with grade I. Positive correlation between HPV and EBV in the oral lesions analyzed suggested co-infection.

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Discussão Geral

4 DISCUSSÃO GERAL

A otimização das intervenções preventivas e terapêuticas direcionadas ao câncer da região de cabeça e pescoço depende da identificação dos fatores de risco associados à doença, uma vez que a mesma tem caráter multifatorial (Dhar *et al.*, 2000; Curado; Hashibe, 2009; Jalouli *et al.*, 2012; Radoï; Luce, 2013; Périé *et al.*, 2014). Fatores extrínsecos como o tabaco, o álcool (Du *et al.*, 2000; Figuero-Ruiz *et al.*, 2004; Du *et al.*, 2007; Neville *et al.*, 2009) e a radiação solar (Tsantoulis *et al.*, 2007; Neville *et al.*, 2009; Wilkey *et al.*, 2009) já foram definitivamente associados à etiologia desses tumores. Recentemente, a comprovação da participação de agentes virais na patogenia de alguns tipos de câncer tem sinalizado para a possibilidade de que isso ocorra também com os tumores da região de cabeça e pescoço (zur Hausen, 2002; Shiboski *et al.*, 2005; Mirzamani *et al.*, 2006; Siebers *et al.*, 2008; Kis *et al*., 2009; Salem, 2010; Kabeya *et al.*, 2012; Lingen *et al.*, 2013; Jalouli *et al.*, 2012; Nola-Fuchs *et al.*, 2012; Gupta; Metgud, 2013; Sand; Jalouli, 2014; Périé *et al.*, 2014; Jiang *et al.*, 2015).

O *Human papillomavirus* (HPV) e o *Epstein-Barr Virus* (EBV) são os agentes virais mais frequentemente relatados como possíveis fatores de risco para o câncer de cabeça e pescoço, sendo seu papel bem documentado, respectivamente, no carcinoma de orofaringe (Niedobitek, 2000; El-Mofty; Patil, 2006; Mirzamani *et al.*, 2006; Shillitoe, 2009; Chaturvedi *et al.*, 2011) e no carcinoma indiferenciado de nasofaringe (Young *et al.*, 1988; Pathmanathan *et al.*, 1995; Bar-Sela *et al.*, 2004; Mirzamani *et al.*, 2006; Giannoudis *et al.*, 1995; Dawson *et al.*, 2012; Benson *et al.*, 2014). Entretanto, os estudos ainda são conflitantes e com resultados controversos no que se refere à participação desses vírus na etiopatogenia do carcinoma de células escamosas oral (Kis

et al., 2009; Salem, 2010; Lingen *et al.,* 2013), o que motivou a realização da presente pesquisa.

Os resultados obtidos não evidenciaram associação do HPV e do EBV com os tumores avaliados (carcinoma de células escamosas oral e linfoma), já que, nessas lesões, sua ocorrência não diferiu significativamente daquela do grupo das hiperplasias fibroepiteliais causadas por trauma mecânico. Por outro lado, esse achado confirma a ocorrência de HPV e EBV em lesões da mucosa oral cuja etiologia não está associada a esses vírus, o que concorda com os relatos de que a infecção pode acometer mucosa oral normal (Kis *et al.,* 2009; Gupta; Metgud, 2013; Sand; Jalouli, 2014). Os índices consideráveis de EBV nas hiperplasias fibroepiteliais podem ser explicados pelo caráter de latência vitalícia (Chau *et al*., 2006) de um vírus cujo tropismo inclui o epitélio da mucosa oral (Borza; Hutt-Fletcher, 2002). Já no caso do HPV, é provável que o amplo espectro de detecção de HPV (1, 6, 11, 16, 18 e 31) exibido pelo anticorpo empregado no exame imunoistoquímico, contemplando HPVs de baixo e de alto risco, tenha colaborado com os resultados obtidos. Tal aspecto metodológico diferencia o presente estudo de outros em que a análise imunoistoquímica avalia a sobrexpressão da proteína p16 (El-Mofty; Patil, 2006; Lingen *et al.,* 2013; Bussu *et al.,* 2014).

Os carcinomas de células escamosas mais agressivos (graus II e III) exibiram associação com o sexo masculino e com o uso de álcool e tabaco, o que concorda com os relatos da literatura (Du *et al.,* 2000; Figuero *et al.,* 2004; Du *et al.*, 2007; Curado; Hashibe, 2009; Benowitz *et al.,* 2012; Gupta; Metgud, 2013), não tendo exibido, entretanto, qualquer associação com chimarrão ou comorbidades. A faixa etária dos pacientes também não diferiu significativamente entre os grupos. As correlações avaliadas, por sua vez, exibiram alguns resultados esperados como a correlação positiva entre Ki-67 e tamanho da lesão, ou já relatados pela literatura, como a correlação inversa

entre tamanho da lesão e HPV (Zheng *et al*., 2010; Rischin *et al*., 2010), ou ainda a correlação positiva entre HPV e EBV(Mirzamani *et al.*, 2006; Jalouli *et al.*, 2010; Jalouli *et al.*, 2012; Khenchouche *et al*., 2013; Jiang *et al.*, 2015). Esta última sugerindo a existência de coinfecção pelos agentes virais estudados.

Amostras de distintas procedências, seja geográfica no que se refere aos pacientes, ou anatômica em relação à localização dos tumores, bem como diferentes métodos de detecção para HPV e EBV e subjetividade na interpretação dos mesmos são fatores que contribuem para a considerável divergência entre os resultados dos estudos relatados na literatura (Gupta; Metgud, 2013; Pradidarcheep *et al*., 2008). Além disso, as elevadas taxas de prevalência de infecção e o caráter cosmopolita do HPV e do EBV contribuem com a dificuldade em se definir sua participação na etiopatogenia do câncer de boca. Cuidados metodológicos como fatores de inclusão/exclusão, avaliação da interferência de fatores clínicos como idade e sexo dos pacientes, hábitos e comorbidades, bem como a aplicação de um método quantitativo na análise imunoistoquímica, constituíram tentativas de minimizar possíveis vieses. Entretanto, a despeito disso, fatores como o tamanho reduzido da amostra e limitações inerentes à técnica imunoistoquímica podem ter influenciado os resultados obtidos no presente estudo.

A busca de alternativas preventivas, diagnósticas e terapêuticas para o câncer de boca demanda adequado conhecimento dos fatores de risco a ele relacionados, o que também influenciará de forma significativa o prognóstico da doença. Nesse contexto, novas pesquisas se fazem necessárias para definir a real participação dos vírus HPV e EBV na etiopatogenia desses tumores. Entretanto, torna-se mandatório respeitar rigorosa metodologia, com controle de vieses e padronização da aplicação de técnicas de elevada acurácia. Resultados fidedignos dessas investigações repercutirão de forma direta na

abordagem preventiva e terapêutica da doença, especialmente respaldando ou não a implantação de campanhas de vacinação direcionadas a prevenir o câncer de boca, a exemplo do que já acontece com o câncer de colo de útero.

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5 REFERÊNCIAS

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Anexos

ANEXO A

Normas para submissão de manuscritos ao periódico *Archives of Oral Biology*

http://www.elsevier.com/journals/archives-of-oral-biology/0003-9969/guide-for-authors

ANEXO B

Comprovante de submissão do manuscrito ao periódico *Archives of Oral Biology*

Submission Confirmation for Involvement of Human papillomavirus (HPV) and Epstein-Barr virus (EBV) in head and neck cancer: etiopathogenesis and treatment implications with focus on squamous cell carcinoma

ees.aob.0.2f8130.6896273f@eesmail.elsevier.com em nome de Archives of Oral Biology [AOB@elsevier.com]
Enviado: terça-feira, 24 de fevereiro de 2015 10:29
Para: Karen Cherubini; karencherubibi66@gmail.com; Karen Cherubini

Archives of Oral Biology
Title: Involvement of Human papillomavirus (HPV) and Epstein-Barr virus (EBV) in head and neck cancer: etiopathogenesis and treatment implications with focus on squamous cell carcinoma
Authors: Vanessa C Jornada, DDS; Maria A Figueiredo, Ph.D.; Fernanda G Salum, Ph.D.; Karen Cherubini, Ph.D.
Article Type: Review Article

Dear Karen,

Your submission entitled "Involvement of Human papillomavirus (HPV) and Epstein-Barr virus (EBV) in head and neck cancer: etiopathogenesis and treatment implications with focus on squamous cell carcinoma" has been received by Archives of Oral Biology.

You may check on the progress of your paper by logging on to the Elsevier Editorial System as an author. The URL is http://ees.elsevier.com/aob/.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal. Please do not hesitate to contact me if you have any queries.

Kind regards,

(On behalf of the Editors)

Archives of Oral Biology

****
For any technical queries about using EES, please contact Elsevier Author Support at authorsupport@elsevier.com

ANEXO C

Normas para submissão de manuscritos ao periódico *Head & Neck*

http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1097-0347/homepage/ForAuthors.html

ANEXO D

Comprovante de submissão do manuscrito ao periódico *Head & Neck*

Manuscript submitted to Head & Neck - HED-15-0368, Authors Copy
onbehalfof+mcrapanz+mdanderson.org@manuscriptcentral.com em nome de mcrapanz@mdanderson.org
Enviado: segunda-feira, 30 de março de 2015 9:29
Para: Karen Cherubini; kebini.ez@terra.com.br

30-Mar-2015

Manuscript number: HED-15-0368

Dear Prof. Cherubini:

We are pleased to receive your manuscript entitled Relationships of Epstein-Barr virus (EBV), Human papillomavirus (HPV), Ki-67, p53 and clinical features in oral squamous cell carcinoma and oral lymphoma by Jornada, Vanessa; Lopes, Tiago; da Silva, Vinicius; Figueiredo, Maria Antonia; Salum, Fernanda; Cherubini, Karen.

We will be sending it out for review shortly.

To track the progress of your manuscript through the editorial process using our new web-based system, simply point your browser to:

https://mc.manuscriptcentral.com/hed

and log in using the following user ID and password:

If you should have any specific deadlines directly related to this manuscript, please let us know as soon as possible.

Please remember in any future correspondence regarding this article to always include its manuscript ID number HED-15-0368.

If you experience problems associated with the submission web site, please contact the Wiley support staff directly at: mcrapanz@mdanderson.org

Many thanks for submitting your manuscript,

Dr. Ehab Hanna
Editor in Chief
Head & Neck

ANEXO E

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 02 de outubro de 2013

O Projeto de: Dissertação

Protocolado sob nº: 0048/13

Intitulado: Associação entre infecção pelos vírus HPV e EBV e neoplasias malignas de boca: análise histomorfométrica.

Pesquisador Responsável: Profa. Dra. Karen Cherubini

Pesquisadores Associados: Vanessa Chidiac Jornada

Nível: Dissertação / Mestrado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *02 de outubro de 2013*

Este projeto deverá ser imediatamente encaminhado ao CEP/PUCRS.

Profa. Dra. Luciane Macedo de Menezes
Coordenadora da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

Av. Ipiranga, 6681, Prédio 06 sala 210
Porto Alegre /RS – Brasil – Cx. Postal:1429
90619-900

Fone/Fax: (51) 3320-3538
e-mail: odontologia-pg@pucrs.br

ANEXO F

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ASSOCIAÇÃO ENTRE INFECÇÃO PELOS VÍRUS HPV E EBV E NEOPLASIAS MALIGNAS DE BOCA - ANÁLISE HISTOMORFOMÉTRICA

Pesquisador: Karen Cherubini

Área Temática:

Versão: 1

CAAE: 23158913.0.0000.5336

Instituição Proponente: UNIAO BRASILEIRA DE EDUCACAO E ASSISTENCIA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 441.276

Data da Relatoria: 29/10/2013

Apresentação do Projeto:

Vide parecer anterior.

Objetivo da Pesquisa:

Vide parecer anterior.

Avaliação dos Riscos e Benefícios:

Vide parecer anterior.

Comentários e Considerações sobre a Pesquisa:

Vide parecer anterior.

Considerações sobre os Termos de apresentação obrigatória:

Vide parecer anterior.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Todas as pendências foram atendidas.

Situação do Parecer:

Aprovado

Endereço: Av.Ipiranga, 6681
Bairro: **CEP:** 90.619-900
UF: RS **Município:** PORTO ALEGRE
Telefone: (513)320--3345 **Fax:** (513)320--3345 **E-mail:** cep@pucrs.br

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

Continuação do Parecer: 441.276

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

PORTO ALEGRE, 30 de Outubro de 2013

Assinador por:
caio coelho marques
(Coordenador)

Endereço: Av.Ipiranga, 6681
Bairro: **CEP:** 90.619-900
UF: RS **Município:** PORTO ALEGRE
Telefone: (513)320--3345 **Fax:** (513)320--3345 **E-mail:** cep@pucrs.br

Apêndices

APÊNDICE A

FICHA PARA COLETA DE DADOS

Nome do paciente:_____ Idade:_____Sexo:____
Profissão:_____ _____Nº da ficha:_____Nº do AP:_____
Tempo de evolução da doença:_____(meses)
Comorbidades:_____
_____
Tabaco _____cigarros/dia há_____anos. Se parou, durante quanto tempo fumou e há quanto tempo parou_____
_____
Álcool ____/dia há_____anos. Se parou, durante quanto tempo fumou e há quanto tempo parou_____
_____
Outros hábitos: _____
_____
Histórico familiar: _____
_____

AVALIAÇÃO CLÍNICA

Localização da lesão:_____
Tamanho:_____
Diagnóstico anatomopatológico:_____
Outras lesões orais: _____
_____
_____
Observações: _____
_____