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HPV-16 DNA DETECTION IN FRESH TISSUE, SALIVA AND PLASMA OF PATIENTS WITH ORAL LEUKOPLAKIA BY REAL TIME PCR

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HPV-16 DNA detection in fresh tissue, saliva and plasma of patients with oral leukoplakia by real time PCR

Dissertação apresentada à Faculdade de Odontologia do Campus de Araçatuba, Universidade Estadual Paulista ‘’Júlio de Mesquita Filho’’- UNESP, para obtenção do título de Mestre em Odontologia - Área de Concentração em Estomatologia.

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Epígrafe

"É preciso tentar não sucumbir sob o peso de nossas angústias, e continuar a lutar."

- (Harry Potter e o Enigma do Príncipe)
J.K. Rowling

Tomo S. Detecção do DNA do HPV-16 em tecido fresco, saliva e plasma sanguíneo de pacientes com leucoplasia bucal pela *Real Time* PCR. [dissertation]- Araçatuba: UNESP- São Paulo State University; 2017.

Resumo

Objetivo: Avaliar a presença do HPV-16 em tecido fresco, saliva e plasma sanguíneo de pacientes com leucoplasia bucal pela *real time* PCR na região noroeste do estado de São Paulo, Brasil. **Pacientes e métodos:** Trinta e sete pacientes com diagnóstico de leucoplasia bucal foram incluídos no estudo. Destes, foram obtidos dados sociodemográficos, clinicopatológicos, estilo de vida e amostras de tecido fresco, sangue e saliva que foram armazenados a -80ºC para posterior análise molecular. Os materiais obtidos destes pacientes foram submetidos à detecção do DNA viral pela técnica da *real time* PCR com sonda específica para o HPV-16. **Resultados:** Dos 37 pacientes incluídos no estudo, 64,8% eram homens e a idade variou de 25 a 82 anos, com uma média de 58,72 anos. Dezesseis pacientes (43,2%) eram idosos e 43,2%, adultos de meia idade, e apenas 13,6%, adultos jovens. A maioria dos pacientes era fumante (72,9%), sendo que 16,3% eram ex-fumantes e 10,8%, não fumantes. Da mesma forma, a maioria (62,2%) era etilista, 21,6%, ex-etilistas e 16,2%, não-etilistas. Vinte e sete por cento das lesões apresentaram algum grau de displasia epitelial. A detecção do HPV-16 pela PCR em tempo real não foi positiva para nenhuma amostra, resultando em um índice de 0% de detecção. **Conclusão:** O HPV-16 não foi identificado na população estudada. No entanto, outros subtipos do HPV de baixo e alto risco podem estar associados à ocorrência de leucoplasia bucal nesta população, o que requer novas investigações. Estudos epidemiológicos mais amplos são necessários para esclarecer a variabilidade geográfica na prevalência do HPV no carcinoma espinocelular de cabeça e pescoço e lesões bucais potencialmente malignizáveis.

Palavras-chave: Leucoplasia, Papillomaviridae, Reação em Cadeia da Polimerase em Tempo Real.

Tomo S. HPV-16 DNA detection in fresh tissue, saliva and plasma of patients with oral leukoplakia by real time PCR. [dissertation]- Araçatuba: UNESP- São Paulo State University; 2017.

Abstract

Objective: To evaluate the prevalence of HPV-16 DNA detection in fresh tissue, saliva and blood plasma from patients with oral leukoplakia by the real time PCR in the northwest region of the São Paulo state, Brazil. **Patients and methods:** Thirty-seven patients diagnosed with oral leukoplakia were included in the study. Sociodemographic, clinicopathologic and lifestyle data, fresh tissue, saliva and blood plasma samples were collected. Biologic material was stored at -80ºC and then submitted to viral DNA detection by the real time PCR technique with a probe specific for HPV-16. **Results:** Of the 37 patients included in the study, 64.8% were men, and the age ranged from 25 to 82 years, with a mean of 58.72. Sixteen patients (43.2%) were elderly, 43.2% were middle-aged adults, and only 13.6% were young adults. Most patients were smokers (72.9%), 16.3% were former smokers, and 10.8% were non-smokers. Most patients (62.2%) were current drinkers, 21.6% were ex-drinkers and 16.2% were non-drinkers. Twenty seven percent of the lesions presented some degree of dysplasia. HPV-16 detection by real-time PCR was not positive for any sample, resulting in a 0% detection rate. **Conclusion**: The HPV-16 was not identified in the population studied. However, other low and high-risk HPV subtypes might be associated to the occurrence of oral leukoplakia in this population, which requires further investigations. Broader epidemiological studies are required to clarify the geographic variability in the prevalence of HPV in head and neck squamous cell carcinoma and oral potentially malignant lesions.

Keywords: Leukoplakia; Papillomaviridae; Real-Time Polymerase Chain Reaction.

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LIST OF ABBREVIATIONS

HPV, Human Papillomavirus

HNSCC, Head and neck squamous cell carcinoma

OPML, Oral potentially malignant lesions

OL, Oral leukoplakia

DNA, Deoxyribonucleic acid

OSCC, Oral squamous cell carcinoma

UNESP, Universidade Estadual Paulista Júlio de Mesquita Filho

FFPE, Formalin fixed paraffin embedded

EDTA, Ethylenediamine tetraacetic acid

IPC, Internal positive control

nPCR, Nested polymerase chain reaction

ISH, *in situ* hybridization

RT-PCR, Reverse transcription polymerase chain reaction

IHQ, Immunohistochemistry

qPCR, Quantitative polymerase chain reaction

SCC, Squamous cell carcinoma

SUMMARY

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HPV-16 DNA detection in fresh tissue, saliva and plasma of patients with oral leukoplakia by real time PCR*

Authors: Saygo Tomo[1], Lígia Lavezo Ferreira[1], Sandra Helena Penha de Oliveira[1], Éder Ricardo Biasoli[1], Kellen Cristine Tjioe[1], Daniel Galera Bernabé[1], Glauco Issamu Miyahara[1*]

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Introduction

High-risk human papillomavirus (HPV) detection has been strongly correlated to head and neck squamous cell carcinoma (HNSCC), especially of the oropharynx[1]. However, the prevalence and the role of this virus in oral malignant and potentially malignant lesions remain source of debate.

Oral leukoplakia (OL) is the most frequent oral potentially malignant lesion (OPML)[2] and is described as "a white plaque or stain that cannot be characterized clinically or pathologically as any other disease"[2,3]. Thus, the diagnosis of OL is established only after excluding other pathological conditions that present as white plaques[2]. OL malignant transformation rates may vary as from 0.13% to 34%[4] highlighting the importance of studying the factors involved on the appearance and progression of this lesion[4].

Tobacco smoking is still regarded as the main risk factor for the occurrence of OL[5]. Indeed, OL is the most frequent oral lesion in tobacco users[6]. Other risk factors implicated on the onset of OL are alcohol drinking and biological agents, chiefly fungi and virus[2]. On the other hand, a significant number of cases of OL are not associated to the classic risk factors. Bisht[7] et al. found that only 46.67% of OL patients were smokers. Liu[8] et al. reported that 66.5% of the patients with OL had never smoked and that 88.5% of them had never ingested alcoholic beverage, alerting to a possible association of some cases of OL with other etiological factors than tobacco smoking and alcohol drinking.

HPV is a strictly double-stranded epitheliotropic DNA virus[9]. More than 200 HPV subtypes have been described and categorized as low and high-risk HPVs, depending on its potential to lead the epithelium to carcinogenesis[9]. HPV

is the most important etiological agent for cervical cancer[9] and its role on the occurrence of HNSCC has been widely investigated[10,11]. For oropharyngeal cancer, HPV-16 and 18 are already shown to be associated with its onset[11]. On the other hand, the role of HPV on the pathogenesis of other oral lesions is still a mystery. The HPV-16 is the most prevalent high-risk subtype found in HNSCC[1]. Nevertheless, conflicting data report a range between 0 and 100% of HPV positive (HPV+) in oral squamous cell carcinoma (OSCC) cases, demonstrating a need for the definition of the role of this virus on oral cancer[12]

In the last years, the interest for detecting HPV in samples of OPML has increased. Syrjänen[13] et al. performed a systematic review of 956 cases of OPML from cross-sectional studies and found a significant association between OL and HPV infection, supporting the hypothesis of HPV as an etiologic factor for OL. However, there are only few studies investigating the presence of HPV exclusively in OL and the results obtained are quite variable[13]. Either the tissue or the material analyzed, the method used to detect HPV, the criteria for sample selection, and the geographic variation of HPV incidence may influence on the detection rates. Therefore, the aim of this study was to evaluate the prevalence of HPV-16 DNA detection in fresh tissue, saliva and blood plasma from patients with OL by real time PCR in the northwest region of the São Paulo state, Brazil.

Patients and methods

Study design and population

This cross-sectional study included patients with oral leukoplakia diagnosed at the Oral Oncology Center, São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil. The inclusion criteria were i. patients with clinically

and microscopically confirmed diagnosis of primary OL, ii. availability of fresh tissue, blood plasma, and saliva for analysis, and iii. patients who agreed to participate in the study. The exclusion criteria were i. patients who received previous treatment for OL and ii. patients who did not accept to participate in the study. A total of 37 eligible patients were selected. This study was approved by the Research Ethics Committee in human studies (process number: FOA-01034/2011). Each patient with clinical diagnosis of OL was submitted to collection of sociodemographic, clinicopathologic and lifestyle data, blood plasma and saliva, and biopsy for diagnosis confirmation and storage of fresh tissue. Formalin fixed paraffin embedded (FFPE) tissue was submitted to microscopical analysis and fresh tissue, blood plasma, and saliva were submitted to DNA extraction and real time PCR for HPV-16 detection (Figure 1).

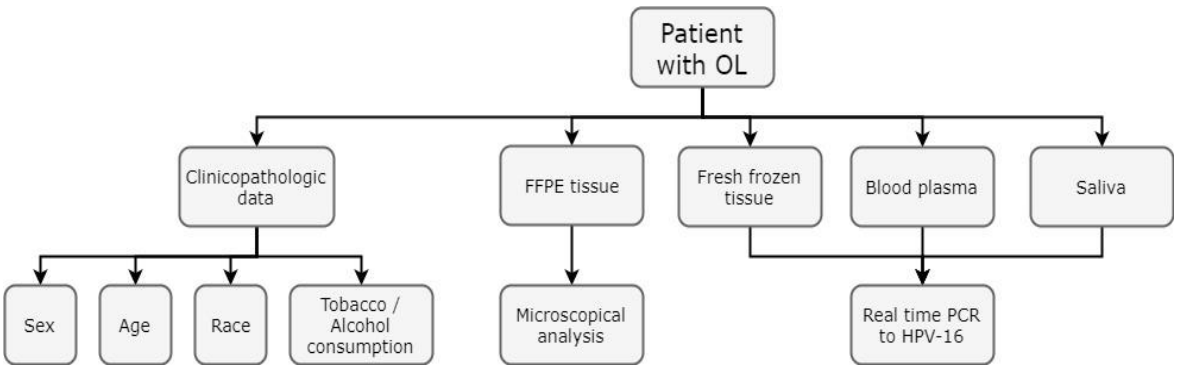

Figure 1. Flowchart of the study. OL: oral leukoplakia; FFPE: formalin fixed paraffin embedded; PCR: Polymerase Chain Reaction; HPV-16: Human papillomavirus 16.

Clinicopathologic data collection

Sociodemographic, clinicopathologic, and lifestyle data of the patients were obtained from their individual records. The sociodemographic variables included age and sex. Lifestyle factors included smoking habit and alcohol consumption. Participants were classified as current, former or nonsmokers /nondrinkers. Current smokers/drinkers were those who reported daily tobacco use for at least one year or reported consumption of any alcoholic beverage at least once in a month, and who continued to smoke and/or drink during their treatment. Former smokers/drinkers included patients who discontinued their use at least one year before diagnosis. Nonsmokers were individuals who have never smoked daily for more than one year, and nondrinkers were considered those patients who had never consumed one drink or more per month for one year. When applicable, additional questions were asked about the usual amount of tobacco and alcohol consumption by type and duration (in years) of the habits. The average number of cigarette equivalents smoked/day was categorically defined as light (≤19), moderate (20–39), and heavy (≥40). Measurements of alcohol consumption was graded as: light (0-2 drinks/day), moderate (3-4 drinks/day), and heavy (>4 drinks/day)[14].

Biological sample collection

When the biopsy was performed, the specimen was divided into two fragments: the first one was fixed in 10% formalin for microscopical analysis, and the second one was immediately frozen at -80°C after surgery for further processing.

For the collection of saliva, patients were instructed not to consume food and beverage 30 minutes before the procedure. Each patient was asked to spit the saliva in a sterile 15mL tube during 10 minutes in order to obtain a minimum of 5mL of saliva. The total saliva obtained was stored at -80ºC for subsequent analysis.

Blood samples were collected before the biopsy and at the beginning of the treatment. The blood was collected via venipuncture, deposited in a tube containing ethylenediamine tetraacetic acid (EDTA), and immediately centrifuged at 1.500 rpm and 4°C for 20 minutes. The plasma was collected and stored at -80°C for further analysis.

Laboratory Procedures

DNA extraction

Total DNA was extracted from tissue, saliva and blood plasma samples using a commercial kit QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, samples were inserted in a 1.5mL microtube and treated with 180µL ATL buffer and incubated with 20µL proteinase K per 25mg of sample at 56°C for 3 hours. When tissues were completely lysed, 200µL AL buffer was added with 200 µL ethanol following a cycle of retained centrifugations and addition of buffer solution. The lysate tissue was transferred to the QIAamp Mini spin column washed and then the eluted genomic DNA was collected. DNA concentration and DNA quality were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE 19810, USA), and quantified by reading at absorbance 260nm. DNA was stored at -20°C until further testing.

Detection HPV-16 DNA by real time PCR

Presence or absence analysis for HPV-16 by real time PCR was performed using the TaqMan kit for exogenous internal positive control reagent (Life Technologies) with the HPV-16 TaqMan detection assays (Life Technologies) and Taqman Universal Master Mix (Life Technologies). According to the manufacturer's instructions for each reaction, 10μL of Universal TaqMan Master mix, 2μL of 10x Exo IPC Mix, 0.4μL of 50x Exo IPC DNA, 1μL of TaqMan assays for HPV-16 and 4.6μL of deionized water, composing the PCR mix. In each well it was added 18μL of the previously prepared mix and 2μL of 10x Exo IPC Block or 1x TE or sample. In addition, a SiHa (a cell line with integrated HPV-16) sample was used for exogenous positive control. The reaction was performed in the standard time stipulated by the Step-One Plus equipment (Life Technologies).

Statistical Analysis

Data were summarized in Excel 2013 software (Microsoft, Redmond, WA) and statistical analysis was performed using Epi Info 7 software (Centers for Disease Control and Prevention, Atlanta, GA). Absolute numbers and frequency distributions were provided for variables of interest and mean values with standard deviation were provided for age (years), smoking and alcohol consumption duration (years). Missing data were excluded from calculations.

Results

Of the 37 patients included in the study, 24 (64.8%) were men and 13 (35.2%) were women. The mean age was 58.72 years. Sixteen (43.2%) individuals were middle-aged (45-59-years old), 16 (43.2%) were elderly (>60-years old), while only 5 (13.5%) patients were young-adults (<45-years old). Most of patients were white (66.7%) while 12 (33.3%) were non-white (Table 1).

Regarding lifestyle, most patients were current smokers (72.9%), 6 (16.2%) were former smokers and 4 (10.8%) were nonsmokers. Among patients who had ever been exposed to the habit of tobacco smoking (84.3%), most were considered light smokers (53.2%), while 10 (31.2%) were moderate smokers, and only 5 (15.6%) were heavy smokers. Twenty-three (62.2%) patients with OL were current alcohol drinkers, eight (21.6%) were former-drinkers and 6 (16.2%) never drinkers. Regarding the alcohol consumption, most patients were light drinkers (57.6%), while 10 (38.4%) patients were moderate drinkers, and one (3.8%) was a heavy drinker (Table 1).

Seventy two percent of the OL did not exhibit cellular atypia, 7 (18.9%) patients had low and 3 (8.2%) had moderate epithelial dysplasia. No patient had severe epithelial dysplasia (Table 1).

DNA extraction was successful in all fresh tissue, saliva, and blood plasma samples of the 37 patients. HPV-16 DNA amplification by real time PCR was negative for all biological sources from all patients (Supplementary figure 1 and 2, Table 1).

Table 1. Sociodemographic, clinicopathologic and lifestyle characteristics and HPV-16 detection results of patients with oral leukoplakia.

VARIABLE	N	%
Sex		
Male	24	64.8
Female	13	35.2
Age		
Young	5	13.5
Middle age	16	43.2
Elderly	16	43.3
Tobacco smoking status		
Nonsmoker	4	10.8
Current smoker	27	72.9
Ex-smoker	6	16.3
Tobacco smoking intensity*		
Light	17	53.2
Moderate	10	31.2
Heavy	5	15.6
Alcohol drinking status		
Nondrinker	6	16.2
Current drinker	23	62.2
Ex-drinker	8	21.6
Alcohol drinking intensity*		
Light	15	57.6
Moderate	10	38.5
Heavy	1	3.8
Epithelial dysplasia		
Absent	27	72.9
Low	7	18.9
Moderate	3	8.2
Severe	0	0
HPV-16 DNA in fresh tissue		
Positive	0	0
Negative	37	100
HPV-16 DNA in saliva		
Positive	0	0
Negative	37	100
HPV-16 DNA in blood plasma		
Positive	0	0
Negative	37	100
TOTAL	**37**	**100**

*Excluding patients with missing data.

Discussion

Currently HPV-16 virus is considered a well-recognized risk factor for oropharyngeal cancer[1] and it seems to predict a better prognosis of the disease[15]. Differently, the presence of high-risk HPV subtypes in oral cancer has been reported as high as 54.3 - 94.7%[16,17] in the first studies but recent investigations have found low or even absent prevalence of HPV in OSCC[18,19]. These controversial findings led us to wonder if the HPV influences the process of carcinogenesis.

Since 1977, when Fejerskov[20] et al. suggested the participation of viral infection on the pathogenesis of OL, the most frequent premalignant lesion, huge efforts have been dedicated to better understand this possible association. To date, in the last 10 years only 17 studies investigated this matter and they provided highly variable epidemiologic evidence regarding the detection of HPV in OL (Table 2).

Table 2. Reported prevalence of HPV in oral leukoplakia in the last 10 years.

N	Author, country	Year	Sample	Method	HPV+ (%)	HPV-16+ (%)
1	Koyama[21] et al., Japan	2007	FFPE[α] tissue	PCR*	73%	-
2	Khovidhunkit[22] et al., Thailand	2008	FFPE[α] tissue or fresh tissue	*n*PCR[±]	0%	-
3	Llamas-Martinez[23] et al., Spain	2008	FFPE[α] tissue	PCR*	45.7%	40%
4	Khanna[24] et al., India	2009	FFPE[α] tissue	ISH[£]	40%	33%
5	Yang[25] et al., Taiwan	2009	FFPE[α] tissue	*n*PCR[±]	22.8%	-
6	Szarka[26] et al., Hungary	2009	Exfoliated cells	*RT*-PCR[β]	40.9%	27.27%
7	Mathew[27] et al., India	2011	Exfoliated cells	PCR*	65%	10%
8	Baig[28] et al., Pakistan	2012	Exfoliated cells	PCR*	19.2%	-
9	Kristoffersen[29] et al., Norway	2012	FFPE[α] tissue	*n*PCR[±]	64%	0%
10	Prakash[30] et al., India	2013	FFPE[α] tissue	IHQ[£] (p16)	57.1%	-
11	Sikka e Sikka[31], India	2014	FFPE[α] tissue	PCR*	45%	-
12	Pierangeli[32] et al., Italy	2015	Exfoliated cells	*q*PCR[Ω]	33.3%	66.6%
13	Saghravanian[33] et al., Iran	2015	FFPE[α] tissue	PCR*	0%	-
14	Bhargava[34] et al., India	2016	FFPE[α] tissue	*RT*-PCR[β]	0%	0%
15	Ramya[35] et al., India	2017	Fresh tissue	PCR*	20%	-
16	Bhosale[36] et al., India	2017	FFPE[α] tissue	IHQ[£] (p16) + *n*PCR[±]	0%	0%
17	Ferreira[37] et al., Brazil	2017	Fresh tissue	*n*PCR[±]	68.7%	-
18	Tomo et al., Brazil	2018	Fresh tissue	*qPCR*	-	0%

[α]Formalin fixed paraffin embeded tissue, *Polymerase Chain Reaction, [±]Nested PCR, [£]Immunihistochemestry [β]Reverse Transcription PCR, [Ω]Quantitative PCR.

Here, to the best of our knowledge, we reported for the first time the incidence of HPV-16 in fresh tissue, blood, and saliva from the same patient with OL collected at the same time.

We found a null prevalence of HPV-16 in our OL samples using a highly accurate method. As one can see on Table 2, the reported incidence of HPV-16 on OL is quite variable (0-66.6%). Such difference may be attributed to different methods employed to detect HPV[38]. The amplification of viral DNA through PCR in its varieties has shown the highest sensitivity and specificity[38,39]. In the present study, the real time PCR with specific probe to HPV-16 DNA was used. Despite of its high accuracy, only our and two additional studies used real time PCR for HPV detection in OPML[26,34]. Bhargava[34] et al did not observe the HPV-16 in OL fresh tissue, corroborating our findings while Szarka[26] et al found 27.27% of HPV-16 positivity in exfoliated cells of OL, a different biological source from ours.

The studies in which the HPV was present in high rates of OL cases[27,30,37], employed different techniques such as conventional PCR, *in situ* hybridization, and immunohistochemistry to the protein p16[30,36]. Such approaches are considered effective methods for the detection of HPV in oral lesions but may lead to false positive results due to its relatively lower specificity[39].

In addition, the source of material obtained from patients may also influence the results. The use of fresh tissue for the detection of the virus is the most desirable option, however, due to the necessity of such tissue for the diagnosis and histopathological grading of the lesions and the fact that it is an invasive surgical procedure to which not all patients are prone to be submitted, such material may not always be available for viral detection[40]. Biological materials such as saliva and blood plasma have been investigated as sources for HPV detection in patients with HNSCC[40]. However, the feasibility of using saliva and blood plasma to detect HPV in potentially malignant lesions as

leukoplakia was not widely explored as for HNSCC[40,41]. In a recent study from our group, plasma and saliva were validated as sources of biological materials for HPV detection by nested PCR (nPCR) in patients with OL[37]. In the present study, although HPV-16 was not detected in any sample, there was a 100% concordance rate between fresh tissue, saliva, and blood plasma.

Variable prevalence rates of HPV in patients with HNSCC and OPML led researchers to deepen investigations into its epidemiological aspects. Several studies documented that the presence of HPV in HNSCC appears to demonstrate geographical variability. Recently, the prevalence of HPV-16 infection in oropharyngeal squamous cell carcinoma (OPSCC) in the USA was 60%, in Europe 31%, and in Brazil only 4%[19]. Piña[42] et al. observed a null prevalence of HPV in samples from tonsil SCC in Brazil. This is in contrast with results found by Betiol[43] et al. (19.4% of HPV+ HNSCC), and Petito[44] et al. (25% of HPV+ oral and oropharyngeal SCC) in Brazilian samples. However, in a recent study conducted in our center HPV was not detected in any fresh tissue samples from oral and oropharyngeal squamous cell carcinoma (SCC) by linear array assay (unpublished data).

Although the paucity of studies in Brazil analyzing the prevalence of HPV in OL, the absence HPV-16 observed in the samples of the present study is in accordance with further evidence from Thailand[22] and India[33,34,36]. In addition, Krostoffersen[29] et al. observed in Norway that the HPV was detected in 64% of OL samples but none were HPV-16+. On the other hand, we previously found a HPV prevalence of 68.7%[37] in patients with OL by nPCR. Such conflicting data reinforces that the heterogeneity of patients' samples and methods to detect the HPV impairs the comparison of different studies and solid conclusions are still difficult to be drawn.

In this study 72.9% of the patients with OL were current smokers and only 10.8% were never smokers. The association between smoking and the prevalence of HPV in the oral mucosa and SCC and OPML has been controversial. It is known that smoking induces an increase in the keratinization of the epithelium[45], protecting the oral mucosa from secondary trauma and providing a greater resistance against HPV infection[25]. On the other hand, immunosuppression caused by smoking has been a factor considered in the studies that associate the prevalence of HPV in the mouth with smoking[46,47].

Kero[46] et al. showed that after a 7-year follow-up period the permanence of HPV in the oral mucosa of men was significantly associated with the habit of smoking, corroborating the results by Haukioja[47] et al., who observed a significant relationship between persistent HPV infection and smoking among women ($p < 0.05$). In contrast, Yang[25] et al. demonstrated that in a sample of 124 smokers with OL, there was no significant relationship between HPV infection and smoking ($p = 0.21$). In addition, Sikka and Sikka[31] concluded that although tobacco is the most important etiological factor in the occurrence of OL, other factors, including HPV, may play a fundamental role in the pathogenesis of a plot of cases. A previous study from our laboratory showed that most patients with HPV+ SCC of the floor of mouth were not smokers[48], which was statistically confirmed in a later study, in which the degree of smoking was high in patients with (HPV-) OSCC ($p = 0.02$)[49]. Therefore, we agree with Reed[50] et al. who suggested that studies and analyzes must be conducted to elucidate the joint role of these two factors in the pathogenesis of potentially malignant and malignant oral lesions.

Within the limits of this study, the following conclusions can be drawn. The HPV-16 was not identified in the population studied. However, other low

and high-risk HPV subtypes might be associated to the occurrence of OL in this population, which requires further investigations. Broader epidemiological studies are required to clarify the geographic variability in the prevalence of HPV in HNSCC and OPML.

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Conflict of interest statement

The authors declare that there is no potential conflict of interest regarding this work.

Founding

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Anexo A

Gráficos dos ensaios de presença e ausência para DNA do HPV-16 pela *Real-Time* PCR

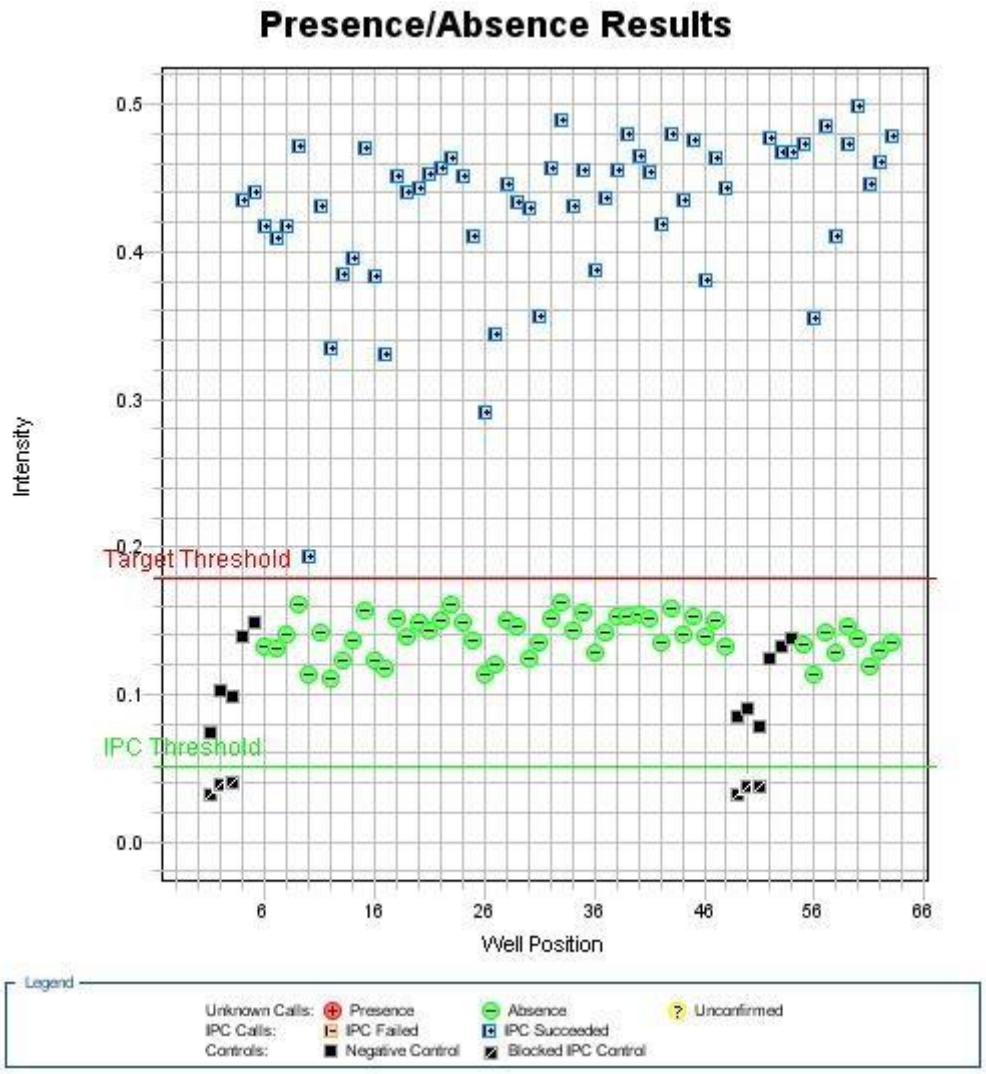

Figure 1. Qualitative real-time PCR results for HPV-16 detection in fresh tissue and saliva samples from patients with OL evidencing samples with absence of viral DNA (green), the positive internal control (IPC) for each reaction (blue) and the three types of negative control (black).

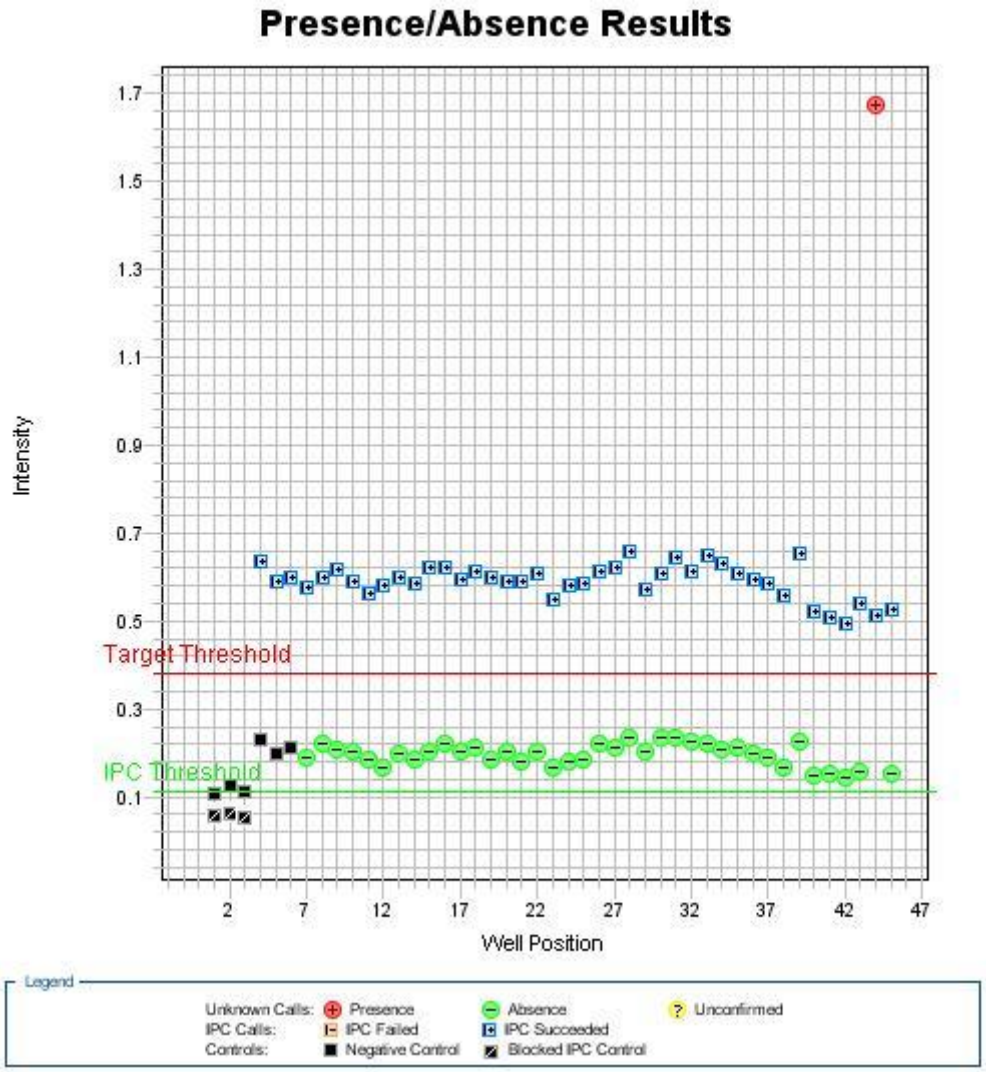

Figure 2. Qualitative real-time PCR results for HPV-16 detection in blood plasma samples from patients with OL evidencing the SiHa cell line sample, used as a positive control (red), samples with absence of viral DNA (green), the positive internal control (IPC) for each reaction (blue) and the three types of negative control (black).

Anexo B

Parecer consubstanciado do CEP – FOA/UNESP

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

COMITÊ DE ÉTICA EM PESQUISA

CERTIFICADO

Certificamos que o Projeto ***"Detecção do HPV por nPCR em leucoplasias bucais: Estudo caso-controle"***, sob a responsabilidade do Pesquisador **GLAUCO ISSAMU MIYAHARA**, está de acordo com os Princípios Éticos em Pesquisa e foi aprovado em 27/05/2011, de acordo com o Processo FOA-01034/2011.

Araçatuba, 06 de junho de 2011.

ALESSANDRA MARCONDES ARANEGA
Vice-Coordenadora do CEP

AMA/sbm.

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Anexo C

Periódico de interesse para submissão

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