CORRESPONDENCE

HPV 18 prevalence in oral mucosa diagnosed with verrucous leukoplakia: cytological and molecular analysis

INTRODUCTION

Currently more than 200 types of human papillomaviruses (HPV) have been classified, where the low-risk ones are those that are not capable of developing into cancer while the high-risk ones are.[1] Cervical cancer and its preceding lesions have been strongly associated with HPV, mainly through the high-risk 16/18 strains. Due to morphological similarities between the cervical mucosa and the upper aerodigestive tract, it is suggested that HPV be proposed as an aetiological factor in the developing of oral cancer.[2]

In 1978, WHO defined leukoplakia as a white plaque of oral mucosa, not removable by scraping, which cannot be characterised clinically or pathologically as any other disorder.[3] Recent publications have grouped leukoplakia with lesions that precede oral cancer or those that are potentially malignant.[4] HPV's role in the aetiology and malignisation of precancerous oral lesions has been studied; however, the results achieved are still not conclusive. The purpose of this study was to identify the prevalence of HPV 16/18 in patients with verrucous leukoplakia of the oral mucosa, along with a profile of their social demographics.

REPORT

Obtaining samples

The UNIARARAS community, located in Araras, São Paulo, Brazil, was divided in two groups: G1, consisting of 16 samples from eight patients with verrucous leukoplakia, due to some of the patients presenting multiple lesions in their oral mucosa and G2, consisting of 16 patients without lesions in their mucosa.

For making the smear slide, samples were fixed to previously identified glass slides, and *Shorr* staining was carried out to nuclear and cytoplasmatic analysis.

The biomolecular analysis samples were collected by scraping the lesioned mucosa using brushes such as 'Campos da Paz' (cytobrush®) and stored in eppendorfs (Axygen®) with saline solution 0.9% at approximately −20°C.

β-Globin PCR

After the DNA extraction, PCR of the β-globin gene was carried out through the use of PCO3/PCO4 primer pair,[5] which amplifies a fragment 125 bp long.

L1 HPV region PCR

To amplify the L1 HPV region, the *primer* pair GP5+/GP6+[6] was used to produce one fragment of 150 bp long when the viral DNA is present in the sample.

Specific E7 region PCR

HPV 16 and 18 genotyping was carried out with two pairs of specific *primers*[7] for each viral subtype, which amplifies fragments of the HPV E7 region, generating fragments with 108 and 104 bp long, respectively. Negative and positive standards were used in each amplification to ensure the quality of the PCR.

PCR analysis

Amplified DNA fragments were separated by a 10% polyacrylamide gel electrophoresis and the silver-staining procedure was used for the detection of the amplified fragments in the polyacrylamide gel.

Social demographics questionnaires

The patients involved in this study voluntarily answered questions about their social behaviour (alcohol and tobacco consumption, gender, marital status and age).

Statistical proceedings

This study applied the Fisher exact test to achieve the p value (the limit of significance for all analyses was defined as $p<0.05$), SD, OR and CIs (95%). Statistical data were produced using SISA® (Simple Interactive Statistical Analysis) software.

Ethical approval

The present study was approved by the UNIARARAS committee of scientific merit and research ethics under the protocol number 387/2010, and the decision was homologated at the meeting on 8 June 2010. All patients gave permission for use of samples for cytologic and genomic research.

DISCUSSION

Cytological analysis was able to diagnose low-grade squamous intraepithelial lesion and koilocytosis in 100% (8/8) of the G1 patients. Inflammatory cellular alterations were also identified (figure 1).

The β-globin gene was amplified in all samples in order to avoid false negative results and to verify for integrity of DNA samples. Amplification of the viral L1 region diagnosed the presence of the HPV in 100% (8/8) of the G1 patients in at least one lesioned area of the oral mucosa and in 8.75% (3/16) of the G2 patients (OR=23.02, CI 4.344 to 121.977, p=0.0002) (table 1).

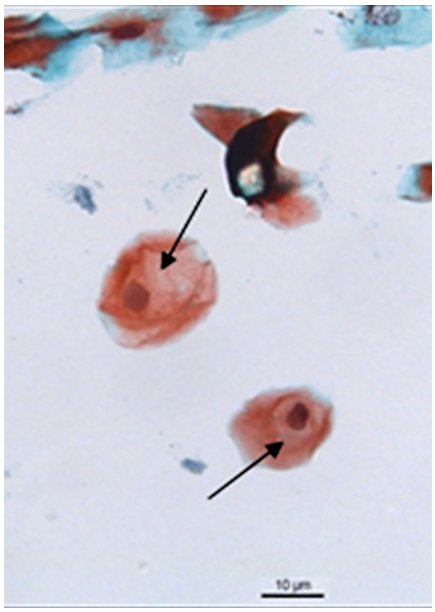

Figure 1 Koylocitosis: 40× micrograph of a koilocyte (Shorr staining). This figure is produced in colour in the online journal—please visit the website to view the colour figure.

With regard to genotyping, none of the patients were diagnosed as HPV 16 positive; however, HPV 18 was present in 62.5% (5/8) of the G1 patients and in 18.75% (3/16) of the G2 patients (OR=7.222, CI 1.076 to 48.476, p=0.0426) (figure 2) (table 2).

The role of the HPV in verrucous leukoplakia is controversial, since there is a wide range in the incidence of virus detection. Association of histopathological diagnosis with PCR methods seems to be a more reliable tool to predict the presence of HPV.

Koilocytosis, diagnosed in all G1 patients, is the most important pathognomonic sign of the HPV infection triggered by viral proteins (E5 and E6),[8] supporting our initial hypothesis: the important viral role in the development of the verrucous leukoplakia.

Considering the results obtained, this study concluded that the HPV is associated with the verrucous leukoplakia of oral mucosa, the viral subtype 18 being the most prevalent in this lesion among the analysed

Table 1 Fisher's exact test: human papillomaviruses (HPV) DNA presence in patients with verrucous leukoplakia and in patients without verrucous leukoplakia

	Verrucous leukoplakia				
HPV DNA	Absent	Present	Total	OR (95% CI)	p Value
Absent	13	0	13	**23.02 (4.344 to 21.977)**	**0.0002**
Present	3	8	11		
Total	16	8	24		

Significance difference at 5%, p value obtained by Fisher's test. Null hypothesis was applied.

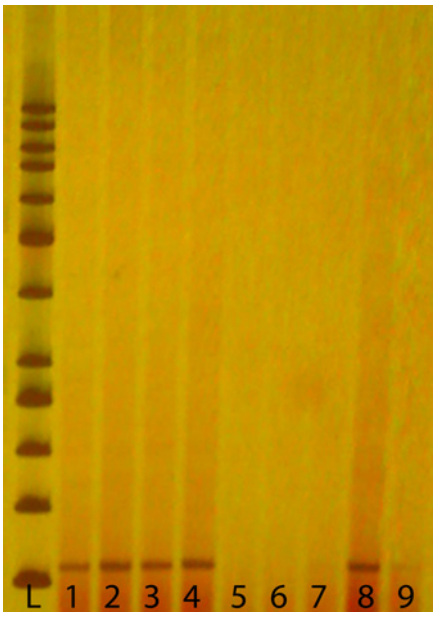

Figure 2 Polyacrylamide gel: L (ladder); samples 1, 2, 3 and 4 human papillomavirus (HPV) 18 positive; samples 5, 6 and 7 HPV 18 negative; sample 8 positive control; sample 9 negative control. This figure is produced in colour in the online journal—please visit the website to view the colour figure.

patients of the group 1. Subtype 16 did not show any association with the lesion in this study. Notwithstanding 18.75% of the group 2 are positive for HPV 18, the virus did not cause any change at the cellular level, possibly due to the virus latency period or to the immune competence of the patient. Among the patients analysed, the social demographic profile in which the verrucous leukoplakia is associated with the HPV 18 in higher proportion, 80% (4/5), are married men, smokers and with alcoholic habits, with average age of 59.75 years.

Table 2 Fisher's exact test: human papillomavirus (HPV) 18 DNA presence in patients with verrucous leukoplakia and in patients without verrucous leukoplakia

	Verrucous leukoplakia				
HPV18 DNA	**Absent**	**Present**	**Total**	**OR (95% CI)**	**p Value**
Absent	13	3	16	**7.222 (1.076 to 48.476)**	**0.0426**
Present	3	5	8		
Total	16	8	24		

Significance difference at 5%, p value obtained by Fisher's test.

Take-home messages

- HPV 18 seems to be associated to Verrucous Leukoplakia in oral mucosa, once viral prevalence was statistically significantly among the samples analyzed.
- HPV 16 didn't show any associations with the Verrucous Leukoplakia, however, study with a larger number of patients should be conducted, to confirm this exclusion.
- Alcoholic and smoking habits seem to be an important factor in the association between HPV and Verrucous Leukoplakia.

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Contributors JCB and JSRB were the principal investigators, and they take primary responsibility for the paper. SK provided the samples and was responsible for the clinical diagnosis. JCB, WT and ACA were responsible for molecular analysis and clinical information collection. SKSS and RB conducted the cytological analysis.

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Competing interests None.

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Notes