Acta Clin Croat 2017; 56:369-374 Original Scientific Paper

10.20471/acc.2017.56.03.02

HUMAN PAPILLOMAVIRUS AS A POTENTIAL RISK FACTOR FOR ORAL PREMALIGNANT LESIONS

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SUMMARY – Oral premalignant lesions (OPLs) and numerous alterations of oral mucosa remain unsolved due to their complex etiopathogenesis. Human papillomaviruses (HPVs), in particular, have been reported as the possible risk factors or cofactors. The aim of the study was to determine the association of different HPV types with oral premalignant lesions, and the potential role of smoking and alcohol use. Eighty patients (mean age ± SD, 52.45±5.56) of both genders, 19 (23.75%) male and 61 (76.25%) female, were enrolled in the study. Study group included 40 patients diagnosed with OPLs (leukoplakia, erythroplakia, actinic keratosis and lichen planus), while control group included another 40 patients with healthy oral mucosa. Genotyping of the HPV types was performed by qualitative real-time HPV typing polymerase chain reaction test. HPV DNA was detected in 30% (12/40) of study group patients and 2.5% (1/40) of control group patients. The results revealed the presence of HPV16 in 15% (6/40), HPV56 in 10% (4/40), and HPV18 in 5% (2/40) of study group cases, and HPV31 in 1 (2.5%) control group patient. The association of oral HPV positivity and smoking/alcohol use in the study group was not statistically significant ($p>0.05$). In conclusion, high-risk HPV types are associated with oral premalignant disorders. However, it remains unknown whether HPV acts as an innocent bystander or it has a role in initiating development of premalignant lesions. Smoking and alcohol use were not associated with the existing oral HPV infection.

Key words: *Mouth diseases; Papillomaviridae; Human papillomavirus 16; Polymerase chain reaction; Macedonia*

Introduction

Oral premalignant lesions (OPLs) such as leukoplakia, erythroplakia and lichen planus have been strongly associated with alcohol and tobacco use, increasing the risk of developing malignancy, although age, trauma and poor oral hygiene are pointed out as well[1-3]. In the last decades, researches emphasize the role of infective agents and of human papillomaviruses (HPV) in particular as the probable risk factors or co-factors in the etiology of these entities. The possible association of OPLs and oral squamous cell cancer with HPV, as reported by Syrjänen *et al.*[4], signifies the establishment of a new field of research. The role of HPV was additionally confirmed by verifying the presence of HPV in oropharyngeal cancers as well[5]. Regardless of different diagnostic methodology, studies pointed to the association of oral lesions and HPV infection. Previous studies report on 80% HPV positive leukoplakias[6], and another study verified HPV DNA in 17.6% of oral leukoplakic lesions and 19.7% of oral lichen planus lesions[7].

Even though it is considered that the group of oral cancers and oropharyngeal cancers are HPV associated, the role of HPV in oral cavity lesions requires further interpretation and higher level of care[8]. Signifi-

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Received June 8, 2016, accepted August 17, 2016

cantly less often, HPV DNA is detected among tobacco smokers and/or chewers[9], and no relationship of alcohol use and HPV positive or HPV negative cases has been detected[10].

In order to estimate the role and association of different HPV types with OPLs, and the association of HPV infection with smoking and alcohol intake, we determined HPV presence by HPV DNA polymerase chain reaction (PCR) and genotyping of the HPV types.

Materials and Methods

Subjects

This case-control, clinical and molecular study was conducted at the Department of Periodontal Disease and Oral Pathology, St. Panteleimon University Dental Clinic in Skopje, Republic of Macedonia. Eighty patients (mean age 52.45, range 39-61) of both genders, 19 (23.75%) male and 61 (76.25%) female, referred to the Department for diagnostic evaluation, were included in the study. Detailed history, medical history and clinical examination were performed in each patient. Forty patients diagnosed with OPLs, mean age 51.87±6.08, were included in the study group. Another randomly selected group of 40 patients, mean age 53.02±4.9, without visible changes of oral mucosa comprised the control group. Included subjects answered a questionnaire containing data on demographic features (age, gender, profession, habits, social conditions, smoking and alcohol use, etc.). All features of oral lesions were recorded, including clinical diagnosis, topography and description. Each lesion was photographed as well.

All included subjects were verbally informed about the research and signed an informed consent form. The study was approved by the Educational and Scientific Board of the Faculty of Dental Medicine, Skopje, Republic of Macedonia.

Molecular analysis

Molecular investigation was performed at the Institute of Public Health, Skopje. The method of exfoliative brush cytology was used for sampling, detection and typing of HPV DNA. Smears were obtained from oral lesions in study group patients and from visually unchanged mucosa of the control group patients using a sterile brush (Kito-Brush, Italy). The mucosa was brushed as suggested by the manufacturer's instructions and then a sterile cotton swab was used in addition. Both the brush and the cotton swab were placed in sterile 15-mL centrifuge tubes (ISOLAB, Laborgeräte GmbH, Wertheim, Germany) containing 1 mL of buffered saline solution with phosphate (PBS). The tubes were frozen within two hours at -20 °C until further processing.

Detection, screening and genotyping of HPV DNA from oral smears were done following the working protocol of the PureLinc™ Genomic DNA Mini Kit (Invitrogen, USA). Consensus primers MY09/MY11 amplifying a fragment from the L_1region of the virus were used for HPV screening. Positive and negative controls were included for each reaction. Genotyping was performed by qualitative real-time HPV typing PCR test, HPV high risk genotyping multiplex real-time PCR test (Sacace Biotechnologies, Italy). The test allows for detection of the 12 most common high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59). The test contains 4 different tubes, each containing primers for detection of specific HPV types, as well as primers for ß-globin genes as internal control.

Descriptive statistics (with mean values and percentages) and Fisher's exact test were used to process study results (Microsoft Excel Data Base and Statistica 7 software). The values of $p<0.05$ were considered statistically significant.

Results

Our study included 80 subjects of both genders, 19/80 (23.75%) male and 61/80 (76.25%) female, divided into two groups as follows: 40 patients diagnosed with OPLs as study group (mean age 51.87±6.08) and 40 randomly selected patients (mean age 53.02± 4.9) without visible changes of oral mucosa as control group. According to clinical examination and clinical diagnosis established by two experienced oral patholo-

Table 1. Distribution of HPV DNA positive and negative cases

	HPV positive (n/%)		HPV negative (n/%)	
Study group	12	30.00	28	70.00
Control group	1	2.50	39	97.50

gists, the OPLs and conditions detected were classified as follows: lichen planus in 77.5% (31/40), leukoplakia in 10% (4/40), actinic (lip) keratosis in 10% (4/40) and erythroplakia in 2.5% (1/40) of patients[11]. Distribution of HPV positive and HPV negative cases is shown in Table 1. HPV DNA was detected in 30% (12/40) of study group patients and 2.5% (1/40) of control group patients (Table 1).

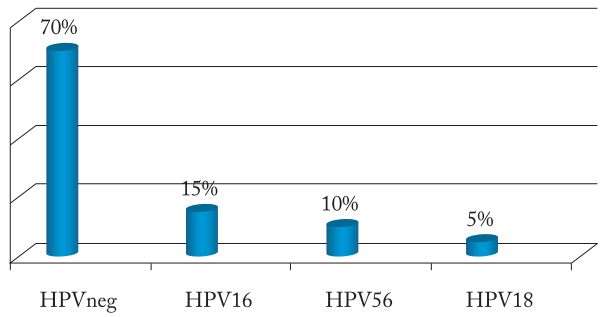

Fig. 1. Distribution of HPV types in the study group.

Distribution of different HPV types in the study group is illustrated in Figure 1. The results revealed the presence of HPV16 in 15% (6/40), HPV56 in 10% (4/40) and HPV18 in 5% (2/40) of patients, while 70% (28/40) of cases were HPV negative.

The association of specified HPV types with certain clinical diagnosis in the study group and control group is shown in Table 2. All HPV positive cases had infection with single HPV type. Out of 80 patients in total, HPV56 was detected in 4 (5%) patients with lichen planus. HPV16 was detected in 6 (7.5%) patients, including 3 (3.75%) patients with lichen planus, 2 (2.5%) patients with leukoplakia and one (1.25%) patient with erythroplakia. HPV18 was detected in 2 (2.5%) patients with leukoplakia, and HPV31 in one (1.25%) control group patient with healthy mucosa. Based on the presented distribution of oral HPV positivity, Fisher's exact test was statistically significant for positive and negative cases from both groups ($p<0.0014$). HPV56 was detected in 2 (2.5%) males, HPV16 in 2 (2.5%) males, and HPV18 in one (1.25%) patient. HPV56 was detected in 2 (2.5%) females,

Table 2. HPV positivity in oral lesions and healthy mucosa

Oral HPV	Lichen planus (n/%)	Actinic keratosis (n/%)	Leukoplakia (n/%)	Erythroplakia (n/%)	Healthy mucosa (n/%)	Total (n/%)
HPVneg	24/30.00	4/5.00	0/0.00	0/0.00	39/48.75	67/83.75
HPV56	4/5.00	0/0.00	0/0.00	0/0.00	0/0.00	4/5.00
HPV16	3/3.75	0/0.00	2/2.5	1/1.25	0/0.00	6/7.5
HPV18	0/0.00	0/0.00	2/2.5	0/0.00	0/0.00	2/2.5
HPV31	0/0.00	0/0.00	0/0.00	0/0.00	1/1.25	1/1.25
Total	31/38.75	4/5.00	4/5.00	1/1.25	40/50.00	80/100.00

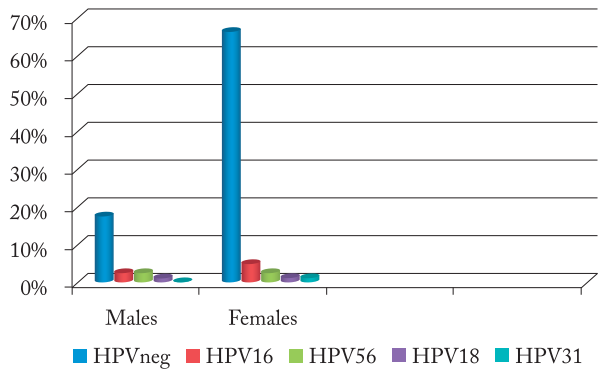

Fig. 2. HPV positivity according to gender.

Table 3. Distribution of patients according to smoking and HPV positivity

Smoking	HPV positivity					Total (n/%)
	HPVneg (n/%)	HPV56 (n/%)	HPV16 (n/%)	HPV18 (n/%)	HPV31 (n/%)	
Yes	18/22.5	1/1.25	2/2.5	1/1.25	0/0.00	22/27.5
No	49/61.25	3/3.75	4/5.00	1/1.25	1/1.25	58/72.5
Total	67/93.50	4/2.00	6/3.00	2/1.00	1/0.50	80/100.00

Table 4. Alcohol users according to HPV positivity

Alcohol	Oral HPV					Total
	HPVneg	HPV56	HPV16	HPV18	HPV31	
Daily	4/5.00	0/0.00	1/1.25	0/0.00	0/0.00	5/6.25
Rarely	63/78.75	4/5.00	5/6.25	2/2.50	1/1.25	75/93.75
Total	67/83.75	4/5.00	6/7.50	2/2.50	1/1.25	80/100.00

HPV16 in 4 (5%) females, HPV18 (1.25%) in one female, and HPV31 in one (1.25%) female (Fig. 2). Fisher exact test yielded no statistically significant gender difference in either positive or negative HPV cases ($p<0.28$).

Results on smoking and HPV positivity are shown in Table 3. Out of 22 (27.5%) smokers, HPV56 was detected in one (1.25%) patient, HPV16 in 2 (2.5%) patients, and HPV18 in one (1.25%) patient. Among nonsmokers (n=58, 72.5%), HPV56 was detected in 3 (3.75%) patients, HPV16 in 4 (5%) patients, HPV18 in one (1.25%) patient, and HPV31 also in one (1.25%) patient.

Distribution of HPV positivity with regard to alcohol use is illustrated in Table 4. Out of 5 (6.25%) patients that declared using alcohol drinks every day, one (1.25%) patient was positive for HPV16. Among patients that rarely used alcohol drinks (n=75, 93.75%), HPV56 positivity was detected in 4 (5%) patients, HPV16 in 5 (6.25%) patients, HPV18 in 2 (2.5%) patients and HPV31 in one (1.25%) patient. Oral HPV positivity was detected in only one (1.25%) patient that reported using alcohol drinks every day.

Discussion

The role of oral HPV infection in malignant alteration of oral premalignant disorders remains unclear. Since a wide array of studies have evidenced and fairly highlighted the role of high-risk HPV in the etiology of oral cancers[12], there are still disagreements about other oral lesions, especially concerning the premalignant disorders and their association with HPV infection. The variability of diagnostic procedures used for detection and typing of HPV has resulted in great differences in the interpretation of the incidence and role of HPV in the etiology of OPLs. Due to discrepancies among geographic areas and study populations regarding particular HPV types[13], interpreting and comparing the results from different regions remains highly challenging.

In our study, HPV DNA was detected in 30% (12/40) of patients diagnosed with OPLs. In the group of patients with healthy mucosa, there was only 1/40 (2.5%) HPV positive case (Table 1). All detected types, including the one from healthy mucosa, belonged to high-risk HPV types (Table 2). The results revealed the presence of HPV16 in 15% (6/40), HPV56 in 10% (4/40), and HPV18 in 5% (2/40) of study group patients (Fig. 1). All positive HPV cases were single HPV infections and we did not detect any case with multiple infection. HPV16 was detected in 7.5% (3/40) of cases with lichen planus, 5% (2/40) of cases with leukoplakia, and 2.5% (1/40) of cases with erythroplakia. HPV18 was detected in 5% (2/40) of cases with leukoplakia and HPV56 in 10% (4/40) of cases with lichen planus. Our results are consistent with literature data disclosing HPV positivity in 17.8% of different oral lesions, premalignant and benign lesions, and 6.8% of HPV positive samples from healthy mucosa as well, with a predominant prevalence of high-risk HPV in potentially malignant disorders (HPV16 and HPV31). Furthermore, the same study highlighted the importance of topography, which is significantly associated with HPV positivity of a particular lesion considering them as the "most frequently exposed sites" to microtrauma[14]. Findings reveal that HPV prevalence increases, being 10% in normal oral mucosa, 22% in benign leukoplakia, and reaching highest value of 46% in oral squamous cell carcinoma[15].

We report on the presence of HPV16 and HPV18 in OPLs, which is consistent with other studies of premalignant and malignant lesions. Furthermore, we verified the presence of HPV56, which also belongs to high-risk HPV types, previously being evidenced in healthy mucosa as well[16]. There are other study reports on the occurrence of the following genotypes in oral mucosa, in a decreasing order of frequency: 16, 6, 18, 56 and 66[17]. So far, we could not find any research con-

cerning the common association of HPV56 with oral lesions, in our case with lichen planus (10%), hence additional studies need to be done to evaluate its appearance and association with lichen planus, the site of appearance, or possibly with this geographic area. Another research reports only one HPV16 positive case (0.98%) in archived paraffin-embedded specimens[18]. Although most of the studies report that HPV18 is one of the most prevalent types in oral lesions, in our study it was detected in 5% of cases, and in another research it was not detected at all[19].

Healthy mucosa is also exposed to HPV infection and the oral HPV predominance in macroscopically normal mucosa is very variable, although the reason has not yet been clarified[20]. According to our results, the prevalence of HPV among healthy individuals is low (2.5%), which is in accordance with other studies[21]. The HPV31 detected in healthy mucosa belongs to high-risk HPV types, demonstrating that even healthy mucosa can be a carrier of high-risk HPV, therefore it is considered that poor oral health may increase the susceptibility to and infectiousness of HPV as an additional risk factor[22].

Research data reveal that tobacco exposure and alcohol intake are significantly associated with prevalent oral HPV infection[23,24]. The association between HPV positivity and the critical risk factors for oral premalignant and malignant diseases, smoking and alcohol intake, was not statistically significant ($p>0.05$) in this study, so HPV infection appears to be an independent manifestation considering these risk factors. However, smoking and alcohol might play an important role in creating proper environment for the acquisition or persistence of the virus, thus studies suggest that smoking may modify either the exposure to the HPV16 virus, the immune response to exposure, or both, but smoking itself does not enhance active infection[25]. This finding was also confirmed by the results of this study.

Even though it remains unclear whether HPV positive OPLs are causally associated with the high-risk HPV types detected or the virus is additionally 'nested', the approach should be the same in view of the nature of the high-risk types.

Herewith, we conclude that high-risk HPV types are associated with oral premalignant disorders. However, it is still unknown whether HPV acts as an innocent bystander, or it has a role in initiating development of premalignant lesions. Smoking and alcohol use is not related to the existing oral HPV infection.

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Sažetak

HUMANI PAPILOMAVIRUS KAO POTENCIJALNI ČIMBENIK RIZIKA ZA ORALNE PREMALIGNE LEZIJE

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Oralne premaligne lezije (OPL) kao i brojne promjene oralne sluznice ostaju neriješene zbog njihove složene etiopatogeneze. Humani papilomavirusi (HPV) osobito su naglašeni kao mogući čimbenici i su-čimbenici rizika. Cilj ove studije bio je utvrditi udruženost različitih tipova HPV s OPL kao i potencijalnu ulogu pušenja i alkohola. U studiju je bilo uključeno 80 pacijenata (srednja dob ± SD, 52,45±5,56 godina) obaju spolova: 19 (23,75%) muškaraca i 61 (76,25%) žena. Skupinu ispitanika s OPL činilo je 40 pacijenata s dijagnosticiranom OPL (leukoplakija, eritroplakija, aktinična keratoza i lihen planus), dok je kontrolnu skupinu činilo 40 pacijenata sa zdravom oralnom sluznicom. Genotipizacija tipova HPV provedena je kvalitativnim *real-time* PCR testom. HPV DNK je otkrivena u 30% (12/40) uzoraka ispitivane skupine pacijenata i 2,5% (1/40) uzoraka kontrolne skupine. Rezultati su pokazali prisutnost HPV16 kod 15% (6/40), HPV56 kod 10% (4/40) i HPV18 kod 5% (2/40) uzoraka skupine s OPL, a HPV31 u jednom (2,5%) uzorku kontrolne skupine. Udruženost pozitivnih nalaza za oralni HPV i navika pušenja/alkohola u ispitivanoj skupini nije bila statistički značajna. U zaključku, visoko rizični tipovi HPV udruženi su s OPL, međutim, ostaje nepoznato djeluje li HPV kao nevini suputnik ili možda ima ulogu u razvoju tih lezija. Pušenje i alkohol nisu povezani s postojećom oralnom HPV infekcijom.

Ključne riječi: *Oralne bolesti; Papillomaviridae; Humani papilomavirus 16; Lančana reakcija polimerazom; Makedonija*