Frequent detection of high human papillomavirus DNA loads in oral potentially malignant disorders

A. Pierangeli[1], F. Cannella[1], C. Scagnolari[1], M. Gentile[1], I. Sciandra[1], G. Antonelli[1], C. Ciolfi[2], C. Russo[2], G. Palaia[2], U. Romeo[2] and A. Polimeni[2]

1) *Virology Laboratory, Department of Molecular Medicine and* 2) *Department of Oral and Maxillofacial Sciences, Sapienza University, Rome, Italy*

Abstract

Human papillomavirus (HPV) is estimated to be the cause of 40–-80% of the squamous cell carcinoma of the oropharynx but only of a small fraction of the oral cavity cancers. The prevalence of oral HPV infection has significantly increased in the last decade, raising concerns about the role of HPV in progression of oral potentially malignant disorders (OPMD) toward squamous cell carcinomas. We sought to study HPV infection in patients with oral lesions, and in control individuals, using non-invasive and site-specific oral brushing and sensitive molecular methods. HPV DNA positivity and viral loads were evaluated in relation to patient data and clinical diagnosis. We enrolled 116 individuals attending Dental Clinics: 62 patients with benign oral lesions (e.g. fibromas, papillomatosis, ulcers) or OPMD (e.g. lichen, leukoplakia) and 54 controls. Oral cells were collected with Cytobrush and HPV-DNA was detected with quantitative real-time PCR for the more common high-risk (HR) and low-risk (LR) genotypes. HPV detection rate, percentage of HR HPVs and HPV-DNA loads (namely HPV16 and in particular, HPV18) were significantly higher in patients than in controls. Lichen planus cases had the highest HPV-positive rate (75.0%), hairy leukoplakia the lowest (33.3%). This study detected unexpectedly high rates of HPV infection in cells of the oral mucosa. The elevated HR HPV loads found in OPMD suggest the effectiveness of quantitative PCR in testing oral lesions. Prospective studies are needed to establish whether elevated viral loads represent a clinically useful marker of the risk of malignant progression.

Keywords: Human papillomavirus, leukoplakia, lichen planus, oral lesions, oral potentially malignant disorders, papillomatosis, squamous cell carcinomas of the oral cavity

Original Submission: 29 April 2015; **Revised Submission:** 23 July 2015; **Accepted:** 10 September 2015

Editor: D. Raoult

Article published online: 25 September 2015

Corresponding author: G. Antonelli, Laboratory of Virology, Department of Molecular Medicine, 'Sapienza' University, Viale di Porta Tiburtina 28, 00185 Rome, Italy

E-mail: guido.antonelli@uniroma1.it

Introduction

Oral cancer is one of the more common cancers worldwide; early detection of precancerous lesions remains a challenge to clinicians [1,2]. Although most oral lesions are benign, many have an appearance that may confound them with oral potentially malignant disorders (OPMD) [1,2]. One of the most common oral lesions is squamous papilloma, an exophytic, pedunculated and usually solitary lesion with small white or red finger-like projections on the surface [1]. Of the oral cavity lesions, leukoplakia, defined as 'a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer' [1,2], is considered an OPMD whereas it is still debated whether lichen planus, a chronic immuno-inflammatory condition, has potential for malignant transformation [1–3]. Although the risk of progression of a single OPMD is low [1–3], the cellular changes and the associated risk factors leading from epithelial atypia to oral cancer are not well defined [2–4]. Hence, there is a need to differentiate and closely monitor those oral lesions at higher risk of developing cancer [1,2].

http://dx.doi.org/10.1016/j.cmi.2015.09.011

A variable fraction (5–25%) of oral cavity cancer cases is deemed to be associated with human papillomavirus (HPV) [5,6]; however, it is not yet clear whether HPV infection acts as a driving force or as a cofactor in oral carcinogenesis. HPVs are small double-stranded DNA viruses that infect epithelial tissues [7]. Mucosal HPV genotypes are classified into low-risk (LR) types that cause only benign lesions and high-risk (HR) types associated with anogenital cancers [7,8]. HPV has been recognized to be also the cause of 40–80% of head and neck squamous cell carcinomas, mostly tonsil and base of the tongue cancers, whose incidence has significantly increased in the last decade [9]. Importantly, an apparent increase in oral HPV prevalence was observed in the general population [10–13], raising concerns about HPV infection and oral cancer risk. In their recent review [12], Chung *et al.* noted that the pooled prevalence of oral HPV infection in healthy individuals between 1997 and 2009 was reported to be 4.5% whereas in a cross-sectional study conducted between 2009 and 2010, oral HPV prevalence increased to 6.9% in the general US population (5579 men and women, aged 14–69 years). More specifically, oral HPV was prevalent in 32% and 16% of human immunodeficiency virus-positive patients and human immunodeficiency virus-negative patients, respectively, all presenting with oral lesions [13]. Given recent global trends of HPV oral infection, the burden of HPV-associated oral cancers is expected to be higher in the next decades.

Oral HPV infection often occurs with no initial signs or symptoms of infection, frequently in the absence of clear risk factors, so that infected individuals are unaware of the infection for a long time. Undoubtedly, many aspects of oral HPV infection remain to be clarified, including modes of acquisition, risk factors, median time to clearance, prevalence of specific genotypes, clinical relevance of high viral loads, and the different outcomes of lesions in relation to the risk of developing oral cancer. In fact, published reports show a marked heterogeneity in patient population, collection methodology, detection technique, diagnostic criteria, anatomical location and selection of control groups [6].

Studying HPV infection and eventually the specific genotype and viral load, in OPMD and other oral lesions, would help in elucidating the role of HPV in oral carcinogenesis and could help to guide future strategies for prevention. To that aim, non-invasive but site-specific methods of collecting oral cells from the lesions should be used. Because of its ease of sampling, a brush biopsy performed with Cytobrush is an adequate technique for diagnostic cytology smears [4,14] to obtain full transepithelial cell samples that are also suitable to be tested for HPV DNA [4]. In view of these considerations and to gain new insights into HPV oral infection, this study assessed the reliability of HPV testing by sensitive and quantitative molecular methods in cells collected by Cytobrush from patients with different types of oral lesions, and from control individuals. In addition, HPV DNA copy numbers were evaluated in positive samples to ascertain whether viral loads were related to HPV risk group, patients' characteristics or clinical diagnosis.

Materials and Methods

Study population

This study was conducted on 116 individuals (62 cases and 54 controls) consecutively enrolled at the Umberto I Dental Clinic in Rome during the period January 2013 to October 2014. Before enrolment, all patients were informed about the procedure and signed an informed consent form. Exclusion criteria were unacceptable oral hygiene because poor oral health is a recognized independent risk factor of oral HPV infection [15]. Individuals enrolled in the study as patients had benign oral lesions (e.g. fibromas, oral traumatic ulcerative lesions), suspected HPV lesions (e.g. papillomatosis) or OPMD (e.g. leukoplakia, lichen planus), whereas the control group was selected from people attending the Dental Clinic for dental conditions without clinically evident lesions in the oral mucosa. Patient data on other localized or systemic diseases and risk factors, e.g. smoking, were recorded.

A brush biopsy was performed applying a Cytobrush (GPS Medical, Bergamo, Italy) with a light pressure and rotating on lesions, to collect a large number of transepithelial cells [14]; brushing was performed inside the whole oral cavity for the control group. The brushing samples were then suspended in 1 ml phosphate-buffered saline and immediately sent to the Virology Laboratory for analysis.

HPV detection

Oral brushings were centrifuged at low speed; the cell pellets then underwent DNA extraction using a QIAamp Blood kit (Qiagen, Hilden, Germany). Amplification of a 400-bp fragment of the human leukocyte antigen (HLA) was initially used to assess the quality of the target DNA, following procedures routinely adopted in the Virology Laboratory for cervical HPV detection and previously described [16]. However, several oral brushes were negative for HLA gene amplification probably because of fragmented DNA [17]. They were therefore unsuitable for amplification with the consensus primer MY09/11 widely used in anogenital HPV detection also in our Laboratory [16]. To overcome this problem, we used HPV type-specific quantitative real-time PCR (qPCR) fluorogenic assays, implemented in a previous study [18], that provide a greater analytical sensitivity without requiring intact genomic DNA. To avoid false-negative results, all samples were tested for the

quality of extracted DNA, amplifying the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in a semi-quantitative way. Accordingly, HPV type-specific primers and TaqMan probes for the more common low-risk (HPV6, 11) and high-risk (16, 18, 31, 33, 53, 58) HPV genotypes were used and copy numbers were measured in samples by means of HPV plasmid external curves [18]. Total DNA concentration was calculated by reading optical density at 260 nm and viral load was determined as HPV copy number per nanogram of total DNA in each sample [18].

Statistical analysis

The chi-square test (two-sided) was used in the statistical analysis of different groups. The non-parametric test for pair-wise comparisons, Mann–Whitney *U* test, was used for analysing viral load values of cases versus controls of LR versus HR and of HPV16 versus HPV18 infections. Statistical tests were considered significant if the p value was $\leq$0.05. Data analysis was carried out with SPSS v.17.0 for Windows.

Results

HPV detection

A total of 116 oral samples were obtained from the Dental Clinic (Table I): 62 were from patients with oral lesions and 54 from controls. Patients' mean age (53.8 years) was higher, though not at a statistically significant level, than that in the control group (40.8 years); attending the clinics for dental conditions is probably more frequent at a younger age.

Total purified DNA concentration median value was 6.9 mg/L (range 1.0–18.9 mg/L); DNA in samples collected from patients was 6.1 mg/L (range 1.0–14.1 mg/L) and from controls was 6.3 mg/L (range 1.2–18.9 mg/L) and the difference was not statistically significant (p > 0.05). All collected samples were positive for semi-quantitative amplification of the GAPDH gene, so that apparently, all results were interpretable with respect to the cellular control.

Overall, 52/116 samples (44.8%) were positive for one of the HPV genotypes tested in the study; no multiple infection was found either in cases or in controls. The HPV positivity rate was higher in the case group (33/62, 53.2%) than in the control group (19/54, 34.5%) at a borderline statistically significant level (p 0.042) with a significantly higher (p 0.027) percentage of HR HPVs in samples from the cases (29/33, 87.9%) than in the control samples (12/19, 63.2%). HPV16 was the most frequently detected HPV genotype in both groups; HPV18, the second most common in cervical cancer, ranked second in patient infections. In the control group, which had more low-risk infections than the patients group, the low-risk HPV6 was the second most common genotype detected (Table I).

Viral loads

In the light of the above results, we sought to evaluate HPV oral infections comparing cases and controls, focusing on type-specific HPV-DNA load in samples calculated as HPV-DNA copy number per nanogram (ng) of total DNA in each sample. Median values in cases and controls are reported in Table I. Interestingly, despite a wide variability, the median number of HPV-DNA copies in oral samples from cases (median cases 1.9×10^4 copies/ng; range: 4.3×10^1 to 1.3×10^7) was significantly higher (p 0.000, Mann–Whitney *U* test) than that from controls (median controls 1.1×10^2 copies/ng; range: 6.5×10^1 to 8.5×10^5) (Fig. 1). Moreover, viral loads of HR HPV from cases (median cases 2×10^4 copies/ng; range: 4.3×10^2 to 1.3×10^7) were significantly higher ($p < 0.0001$, Mann–Whitney *U* test) than those from controls (median controls = 1×10^3 copies/ng; range: 5.2×10^1 to 8.5×10^5) (Table I). In addition, HPV16-positive samples of the patient group had a significantly higher (p 0.003, Mann–Whitney *U* test) median viral load (median cases 1.4×10^4 copies/ng; range: 4.3×10^2 to 6.5×10^6) than that of HPV16-positive controls

TABLE I. Human papillomavirus (HPV) genotype and viral loads in oral samples from case and control groups

	Cases[a] (*n* = 62)	Controls[b] (*n* = 54)	p
Mean age, years (range)	53.8 (17–83)	40.8 (23–75)	n.s.
Gender, male (%)	29 (46.8%)	28 (51.8%)	n.s.
Any HPV (%)[c]	33/62 (53.2%)	19/54 (34.5%)	0.042
HPV genotypes[d]	6 [3],11 [1], 16 [18],18 [6],31 [1],33 [2],53 [1],58 [1]	6 [7], 16 [9],18 [1],33 [1],53 [1]	
HR HPV (%)[c]	29/33 (87.9%)	12/19 (63.2%)	0.027
LR HPV (%)[c]	4/33 (12.1%)	7/19 (36.8%)	
HR HPV viral load, median (range)[e]	2×10^4 (4.3×10^2 to 1.3×10^7)	1×10^3 (5.2×10^1 to 8.5×10^5)	0.000
LR HPV viral load, median (range)[e]	2.8×10^3 (4.3×10^1-4.4×10^3)	4.6×10^2 (6.5×10^1 to 1.7×10^4)	n.s.

[a]Patients attending the Dental clinic with oral mucosa lesions.
[b]Individuals attending the Dental clinic for dental conditions.
[c]Quantitative PCR was performed for the high-risk (HR) types: HPV16, 18, 31, 33, 53, 58 and the low-risk (LR) types: HPV6, HPV11. (%): percentages are the number of cases attributed to HR/LR HPV from the total HPV-positive cases in the group.
[d]Values in square brackets are the number of detections for each genotype.
[e]Viral loads of HR HPV genotypes are median values (range) of determinations expressed as number of copies/ng input DNA in cases and controls.

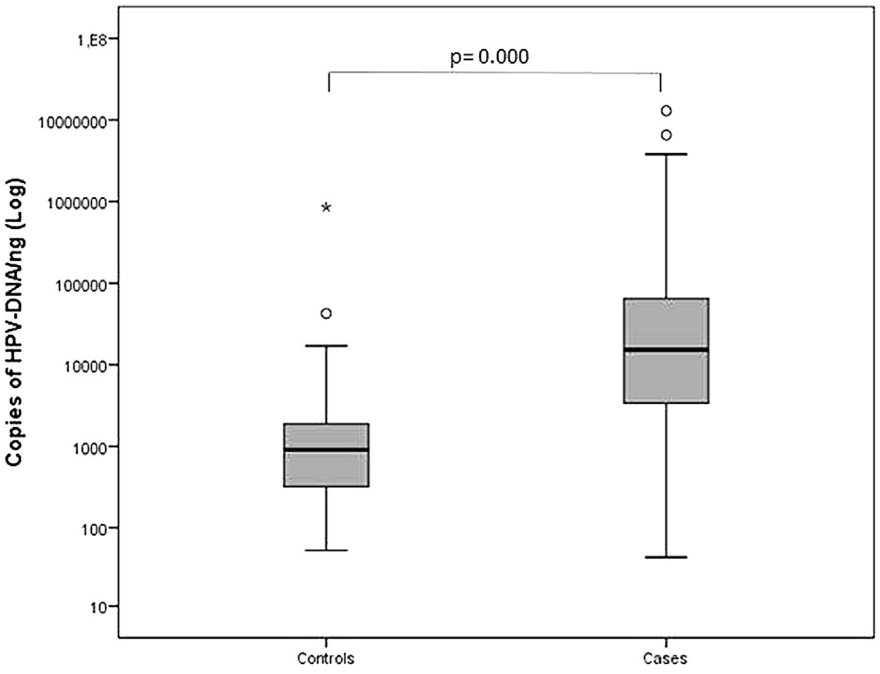

FIG. 1. Median values of type-specific human papillomavirus (HPV) DNA load. Results are cumulative data from 62 patients and 54 controls participating in the study and are expressed as HPV copy number (Log) per nanogram of total DNA in each sample. The non-parametric Mann–Whitney *U* test was used for pairwise comparisons of viral load values between the two groups.

(median controls 9.1 × 10^2 copies/ng; range: 5.2 × 10^1 to 8.5 × 10^5). Of note, HPV18-infected only one individual in the control group whereas HPV18-positive patients had the highest viral loads in lesions (Fig. 2), significantly higher than HPV16 (median DNA copies/ng: HPV18 1.8 × 10^6 versus HPV16 1.4 × 10^4; $p < 0.0001$, Mann–Whitney *U* test).

Patient characteristics and lesions

In the case group, we evaluated HPV results and patient risk factors, clinical diagnosis and affected sites in the oral cavity (Table 2). There was no significant difference between HPV-negative, HR or LR HPV-positive patients in terms of mean age, gender, smoking habit or underlying clinical conditions (Table 2).

The highest HPV infection rates were in patients clinically diagnosed with lichen planus (HPV-positive 9/12 cases, 75.0%), followed by papillomatosis (HPV-positive 14/24, 58.3%), other types of oral lesions, i.e. traumatic ulcers, fibromas, a burning sensation of the oral mucosa (HPV-positive 7/17, 41.2%), and leukoplakia (HPV-positive 3/9, 33.3%) (Table 2). No specific HPV genotype or differences in viral loads were associated with a particular clinical diagnosis or affected site.

Discussion

In normal oral mucosa, reported HPV DNA positivity rates vary considerably among different population groups, but also depend on which oral sample is collected [19–24] as oral rinse and gargle, saliva, oral swabs, oral brushings and biopsies have different oral mucosa cell contents. Oral HPV infection was estimated to be around 7% in US adults [24,25] but ranges from 4.4% when testing only oral rinse [12] to 12.3% when testing oral rinse and oral swabs [22]. In oral cells taken with Cytobrush from Finnish women of reproductive age [26], the rate of HPV positivity was 18–24%, whereas in bioptic samples of normal oral mucosa, HPV positivity was 40% [23].

In this study, to test for HPV infection, a microbrush of the type used in the collection of cervical samples (Cytobrush) was used to collect samples from oral lesions. Several studies have suggested that the Cytobrush collection method permits non-invasive evaluation of potentially malignant lesions by detecting atypia in cells from brush biopsies of lesional material [4,14], which are also suitable for HPV DNA testing [4,20,21]. Using Cytobrush allows the oral mucosa to be tested reaching the non-keratinized strata of the epithelium [14], which is not achieved in oral rinse samples. As a result of the absence of patient discomfort that limits collection of bioptic samples, general dentists and stomatologists could use this technique, which is well-accepted by the patient, also in a screening context, in the absence of overt lesions. In this study, we obtained cells in a comparable amount in both groups, lightly brushing the whole oral cavity in control individuals and directly on the site of clinically evident lesions in the patient group. In patients, brushing the lesions can maximize the chance to detect HPV DNA, which is consistent with the fact that positive

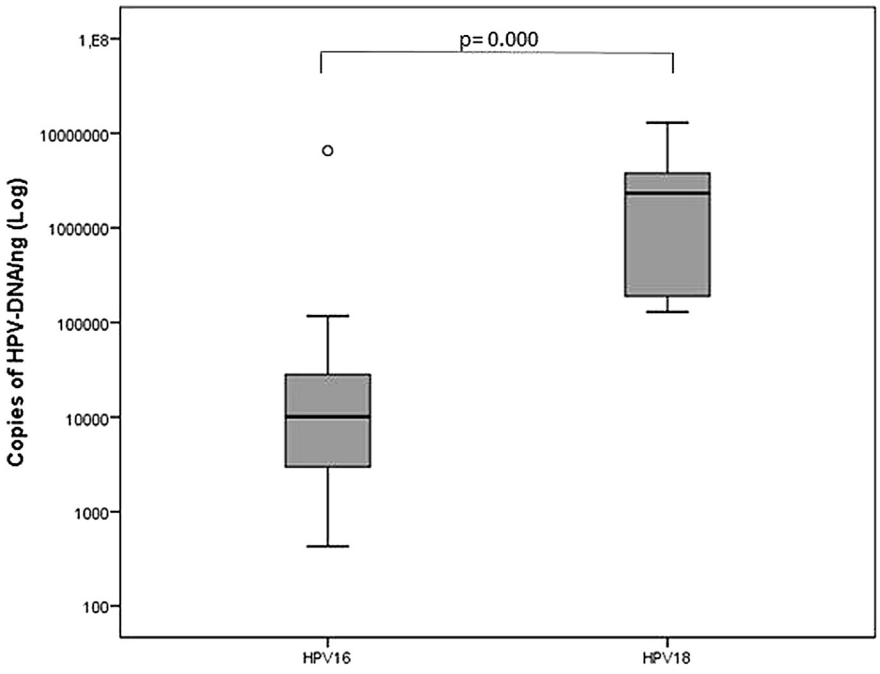

FIG. 2. Median values of type-specific human papillomavirus (HPV) DNA load. Results are cumulative data from HPV16 (n = 18) and HPV18 (n = 9) samples from patients participating in the study and are expressed as HPV copy number (Log) per nanogram of total DNA in each sample. The non-parametric Mann–Whitney U test was used for pairwise comparisons of viral load values between HPV16 and HPV18.

HPV detection on the oral mucosa seems to be associated with lesions in particular anatomical sites [27].

To overcome technical difficulties in HPV detection, we performed type-specific qPCR instead of the nested PCR techniques used in most previous works [19–21,26]. Our PCR assays are specifically designed to yield small amplicons, as appropriate for possibly degraded DNA [17]. Besides, qPCR also has the advantage of being rapid, quantitative, and less prone to contamination. An obvious feature of this method is that, while adding information on the specific HPV genotype, testing all mucosal HPVs in a sample is not routinely feasible. In this study we detected the HR HPV genotypes associated with the higher percentage of cervical cancer cases (16, 18, 31, 33) and the other HPV genotypes (the HR HPVs 53 and 58, and the LR HPVs 6 and 11) more frequently detected in Rome according to our previous paper [17]. Testing a limited number of genotypes can also explain the fact that no multiple infections were detected in this study. In other studies related to oral cavity, HPV multiple infections were in the range of 5–13% [14,23,26], rates apparently lower than in cervical infections.

In this study, the control group's rate of HPV positivity (34.5%) was much higher than in previous Italian studies [20,28] but comparable to recent papers [23,29], so in keeping with the worldwide increase in oral HPV prevalence [12].

To our knowledge, this study is the first to quantify in OPMD and other oral lesions, several HR HPV genotypes other than HPV16, and the LR genotypes HPV6 and 11. Previous investigations either quantified only the overall (i.e. non-type-specific) viral load, or focused on HPV16 so that little is known on the other genotypes. Only one recent study [25] comprehensively quantified HR HPV infections in the oral rinse from a US general population cohort, but did not quantify

TABLE 2. Patient demographic and clinical data stratified according to human papillomavirus (HPV) results

Patient data	HPV-positive (n = 33)	HPV-negative (n = 29)
Mean age, years (range)	52.6 (17–83)	55.1 (21–79)
Male	13	16
Female	20	13
Risk factors[a]		
None	13/29	8/27
Smoking	5/29	8/27
Systemic conditions	12/29	9/27
Non-systemic conditions	5/29	4/27
Clinical diagnosis		
Lichen planus	9 (75.0%) HPV: 6 [1],16 [6], 18 [1],31 [1]	3 (25.0%)
Papillomatosis	14 (58.3%) HPV: 6 [1],16 [6], 18 [3],33 [2], 53 [1], 58 [1]	10 (41.7%)
Leukoplakia	3 (33.3%) HPV: 16 [2],18 [1]	6 (66.7%)
Other lesions[b]	7 (41.2%) HPV:6 [1],11 [1], 16 [4]],18 [1]	10 (58.8%)
Site of the lesion		
Cheek mucosa	3 (30.0%) HPV:6 [1],16 [1],18 [1]	7 (70.0%)
Hard palate	5 (62.5%) HPV: 16 [2],18 [3]	3 (37.5%)
Soft palate	4 (57.1%) HPV:16 [1],31 [1], 33 [1],58 [1]	3 (42.9%)
Oral cavity	2 (40.0%) HPV:16 [2]	3 (60.0%)
Floor of the mouth	2 (50.0%) HPV: 11 [1],16 [1]	2 (50.0%)
Lingual margins and belly	3 (60.0%) HPV: 6 [1],16 [1],18 [1]	2 (40.0%)
Back of the tongue	10 (58.8%) HPV:6 [2],16 [7],53 [1]	7 (41.2%)
Periodontal mucosa	3 (75.0%) HPV:16 [1],18 [1],33 [1]	1 (25.0%)
Retromolar trine	1 (50.0%) HPV: 16 [1]	1 (50.0%)

[a]Risk factors are known for 56 patients out of 62; no significant difference was found between HPV-positive and HPV-negative patients.
[b]Other lesions of the oral cavity include: traumatic ulcers, fibromas, and a burning sensation of the oral mucosa.

LR genotypes. In that cohort [25], older age and current smoking were associated with a high oral load of HR HPV types among individuals with a prevalent infection. These results are not directly comparable with ours, given the differences in the study group, in the sampling techniques, in the number of HR genotypes tested and in the expression of viral load results. The last issue is highly debated in the field because different techniques are used for expressing viral load with respect to the input DNA in the sample and it is difficult to compare different studies. Anyway, interesting differences emerged from our analysis of viral load results: the cases had significantly higher median loads of HR HPV with respect to LR, and of HPV16 with respect to controls, all findings consistent with the reported predominant role of HPV16 in OSCC [30,31]. Nonetheless, our data showed that HPV18 might also play an important role in oral infections with elevated viral loads that could cause a risk of persistence. It has been shown that HPV16 cervical infections (but not those of other HPV genotypes) with high DNA loads are at greater risk of clinical progression [32]. Oral HPV16 load was significantly associated with the time to clearance of oral infections in a recent paper [33]; in this respect, HPV18 oral infections were not addressed.

Comparing our results of HPV detection in the different oral lesions, we noted a very high positivity in lesions clinically diagnosed as lichen planus (75%) whereas the other lesions had comparable rates of HPV positivity with respect to previous reports [20,30,31]. In particular, previous Italian studies detected HPV DNA by nested PCR performed in exfoliated mucosal cells in 17–22% of leukoplakia cases and 20–25% of lichen planus [21,28]. In other recent studies and meta-analyses, HPV detection rates in oral lichen planus range from 10% to 33% [30,31,34,35]; we cannot presently explain this discrepancy with the high HPV detection rates in lichen planus in this study. Lichen planus is a chronic inflammatory autoimmune disease [3] and the frequent ulceration makes this lesion more susceptible to HPV infection. HPV oncogenes in turn may help proliferating epithelial cells to escape apoptosis [7,34,35] but the HPV contribution to the risk of oral cancer is still uncertain. Prospective studies correlating HPV type-specific detection with histology and other host cell biomarkers are needed to clarify this issue.

In conclusion, this study implemented a non-invasive sample collection method with a sensitive and quantitative molecular method, detecting high rates of oral HPV-DNA. The notion of oral HPV infection as a not uncommon condition in normal oral mucosa poses potential for vaccine-based prevention, considering that the vaccine genotype HPV16 was the most frequently detected. It remains to be clarified whether low viral loads in the absence of a clinically evident lesion could be indicative of transient infections. Conversely, HPV infections at elevated viral loads, such those we found in HPV16 and HPV18, could be associated with an increased risk of persistence and progression, to be tested in prospective studies.

Considering the rising incidence of oral cancer, these findings could be relevant to dentists and stomatologists to enhance their knowledge in this area and for clinical patient management.

Transparency Declaration

This study was supported by grants from Sapienza University to AP (Ricerca Scientifica 2012: C26A12FS9N) and to CS (Ricerca Scientifica 2014: C26A14PFJB). The authors declare no conflicts of interest.

References

[1] van der Waal I. Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. Oral Oncol 2009;45:317–23.
[2] Dionne KR, Warnakulasuriya S, Zain RB, Cheong SC. Potentially malignant disorders of the oral cavity: current practice and future directions in the clinic and laboratory. Int J Cancer 2015;136:503–15.
[3] Fitzpatrick SG, Hirsch SA, Gordon SC. The malignant transformation of oral lichen planus and oral lichenoid lesions: a systematic review. J Am Dent Assoc 2014;145:45–56.
[4] Donà MG, Giuliani M, Vocaturo A, Spriano G, Pichi B, Rollo F, et al. Cytology and human papillomavirus testing on cytobrushing samples from patients with head and neck squamous cell carcinoma. Cancer 2014;120:3477–84.
[5] Lingen MW, Xiao W, Schmitt A, Jiang B, Pickard R, Kreinbrink P, et al. Low etiologic fraction for high-risk human papillomavirus in oral cavity squamous cell carcinomas. Oral Oncol 2013;49:1–8.
[6] Walline HM, Komarck C, McHugh JB, Byrd SA, Spector ME, Hauff SJ, et al. High-risk human papillomavirus detection in oropharyngeal, nasopharyngeal, and oral cavity cancers: comparison of multiple methods. JAMA Otolaryngol Head Neck Surg 2013;139:1320–7.
[7] Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol 2002;55:244–65.
[8] Castle PE. The evolving definition of carcinogenic human papillomavirus. Infect Agent Cancer 2009;11:4–7.
[9] Dalianis T. Human papillomavirus and oropharyngeal cancer, the epidemics, and significance of additional clinical biomarkers for prediction of response to therapy (Review). Int J Oncol 2014;44:1799–805.
[10] Edelstein ZR, Schwartz SM, Hawes S, Hughes JP, Feng Q, Stern ME, et al. Rates and determinants of oral human papillomavirus infection in young men. Sex Transm Dis 2012;39:860–7.
[11] Kreimer AR, Pierce Campbell CM, Lin HY, Fulp W, Papenfuss MR, Abrahamsen M, et al. Incidence and clearance of oral human papillomavirus infection in men: the HIM cohort study. Lancet 2013;382:877–87.
[12] Chung CH, Bagheri A, D'Souza G. Epidemiology of oral human papillomavirus infection. Oral Oncol 2014;50:364–9.
[13] Muller K, Kazimiroff J, Fatahzadeh M, Smith RV, Wiltz M, Polanco J, et al. Oral human papillomavirus infection and oral lesions in HIV-positive and HIV-negative dental patients. J Infect Dis 2015 Feb 13. pii: jiv080. [Epub ahead of print].

[14] Poate TW, Buchanan JA, Hodgson TA, Speight PM, Barrett AW, Moles DR, et al. An audit of the efficacy of the oral brush biopsy technique in a specialist Oral Medicine unit. Oral Oncol 2004;40:829–34.
[15] Bui TC, Markham CM, Ross MW, Mullen PD. Examining the association between oral health and oral HPV infection. Cancer Prev Res (Phila) 2013;6:917–24.
[16] Verteramo R, Pierangeli A, Calzolari E, Patella A, Recine N, Mancini E, et al. Direct sequencing of HPV DNA detected in gynaecologic outpatiens in Rome, Italy. Microbes Infect 2006;8:2517–21.
[17] Mirghani H, Amen F, Moreau F, Guigay J, Ferchiou M, Melkane AE, et al. Human papilloma virus testing in oropharyngeal squamous cell carcinoma: what the clinician should know. Oral Oncol 2014;50:1–9.
[18] Pierangeli A, Scagnolari C, Degener AM, Bucci M, Ciardi A, Riva E, et al. Type specific human papillomavirus-DNA load in anal infection in HIV-positive men. AIDS 2008;22:1929–35.
[19] Bouda M, Gorgoulis VG, Kastrinakis NG, Giannoudis A, Tsoli E, Danassi-Afentaki D, et al. "High risk" HPV types are frequently detected in potentially malignant and malignant oral lesions, but not in normal oral mucosa. Mod Pathol 2000;13:644–53.
[20] Giovannelli L, Campisi G, Lama A, Giambalvo O, Osborn J, Margiotta V, et al. Human papillomavirus DNA in oral mucosal lesions. J Infect Dis 2002;185:833–6.
[21] Termine N, Giovannelli L, Rodolico V, Matranga D, Pannone G, Campisi G. Biopsy vs. brushing: comparison of two sampling methods for the detection of HPV-DNA in squamous cell carcinoma of the oral cavity. Oral Oncol 2012;48:870–5.
[22] Edelstein ZR, Schwartz SM, Koutsky LA. Incidence of oral human papillomavirus infection. Lancet 2013;382:1554.
[23] Blioumi E, Chatzidimitriou D, Pazartzi CH, Katopodi T, Tzimagiorgis G, Emmanouil-Nikoloussi EN, et al. Detection and typing of human papillomaviruses (HPV) in malignant, dysplastic, nondysplastic and normal oral epithelium by nested polymerase chain reaction, immunohistochemistry and transitional electron microscopy in patients of northern Greece. Oral Oncol 2014;50:840–7.
[24] Gillison ML, Broutian T, Pickard RK, Tong ZY, Xiao W, Kahle L, et al. Prevalence of oral HPV infection in the United States, 2009–2010. JAMA 2012;307:693–703.
[25] Chaturvedi AK, Graubard BI, Pickard RK, Xiao W, Gillison ML. High-risk oral human papillomavirus load in the US population, National Health and Nutrition Examination Survey 2009–2010. J Infect Dis 2014;210:441–7.
[26] Rautava J, Willberg J, Louvanto K, Wideman L, Syrjänen K, Grénman S, et al. Prevalence, genotype distribution and persistence of human papillomavirus in oral mucosa of women: a six-year follow-up study. PLoS One 2012;7:e42171.
[27] Mravak-Stipetić M, Sabol I, Kranjčić J, Knežević M, Grce M. Human papillomavirus in the lesions of the oral mucosa according to topography. PLoS One 2013;8(7):e69736.
[28] Campisi G, Giovannelli L, Aricò P, Lama A, Di Liberto C, Ammatuna P, et al. HPV DNA in clinically different variants of oral leukoplakia and lichen planus. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;98:705–11.
[29] Ma Y, Madupu R, Karaoz U, Nossa CW, Yang L, Yooseph S, et al. Human papillomavirus community in healthy persons, defined by metagenomics analysis of human microbiome project shotgun sequencing data sets. J Virol 2014;88:4786–97.
[30] Jayaprakash V, Reid M, Hatton E, Merzianu M, Rigual N, Marshall J, et al. Human papillomavirus types 16 and 18 in epithelial dysplasia of oral cavity and oropharynx: a meta-analysis, 1985–2010. Oral Oncol 2011;47:1048–54.
[31] Syrjänen S, Lodi G, von Bultzingslöwen I, Aliko A, Arduino P, Campisi G, et al. Human papillomaviruses in oral carcinoma and oral potentially malignant disorders: a systemic review. Oral Dis 2011;17:58–72.
[32] Gravitt PE, Kovacic MB, Herrero R, Schiffman M, Bratti C, Hildesheim A, et al. High load for most high risk human papillomavirus genotypes is associated with prevalent cervical cancer precursors but only HPV16 load predicts the development of incident disease. Int J Cancer 2007;121:2787–93.
[33] Beachler DC, Guo Y, Xiao W, Burk RD, Minkoff H, Strickler HD, et al. High oral human papillomavirus type 16 load predicts long-term persistence in individuals with or at risk for HIV infection. J Infect Dis 2015 May 7. pii: jiv273. [Epub ahead of print].
[34] Mattila R, Rautava J, Syrjänen S. Human papillomavirus in oral atrophic lichen planus lesions. Oral Oncol 2012;48:980–4.
[35] Szarka K, Tar I, Fehér E, Gáll T, Kis A, Tóth ED, et al. Progressive increase of human papillomavirus carriage rates in potentially malignant and malignant oral disorders with increasing malignant potential. Oral Microbiol Immunol 2009;24:314–8.