Beta-HPV types in patients with head and neck pathology and in healthy subjects

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Abstract

Background

Human papillomaviruses (HPV) are a heterogeneous group of viruses classified into five genera. The beta-HPV type (beta-PV) infection is very common but mostly asymptomatic in immunocompetent individuals. However, beta-PVs play a role in *epidermodysplasia verruciformis* and possibly in non-melanoma skin cancer. Head and neck cancer (HNC) is a common cancer type worldwide and high-risk alpha-PV involvement in HNC has been extensively studied but beta-PV types have rarely been the focus of such studies.

Objectives

To evaluate the prevalence of beta-PV types in HNC, subjects with non-malignant or potentially pre-malignant oral lesions, and healthy controls.

Study design

The frequency of different beta-PVs in samples from oral (n=35) and oropharyngeal (n=35) cancer patients, gender- and age-matched healthy controls (n=70), and subjects with various non-malignant or potentially pre-malignant oral lesions (n=102) was assessed by a highly sensitive, bead-based, multiplex genotyping assay.

Results

Overall, 54.8% of all tested samples contained at least one beta-PV type. Even though the correlation between types found in lavage and tissue specimens from cancer patients was low, there was a large statistically significant difference between oropharyngeal cancer patients and matched controls for HPV5 (P=0.003; OR=15.58) and between both oral (P=0.026; OR=5.7) and oropharyngeal cancer patients (P=0.002; OR=25.5) and controls for HPV122. In

addition, there was no correlation between the prevalence of alpha and beta-PVs in the study patients.

Conclusion

The data describes the prevalence of beta-PVs in HNC. The significant association of HPV122 with HNC might warrant further study as this type has not been extensively studied so far.

Key words:

Oral, oropharyngeal, lesion, cancer, beta-HPV, alpha-HPV

Abbreviations:

HPV, human papillomavirus; beta-PV, beta-genus papillomaviruses; HNC, head and neck cancer; HR-HPV, high-risk HPV; alpha-PV, alpha-genus papillomaviruses; LR-HPV, low-risk HPV; IARC, International Agency for Research on Cancer; SCC, squamous cell carcinoma; TS-MPG, type-specific bead-based multiplex genotyping assay; FFPE, formalin fixed paraffin embedded; PCR, polymerase chain reaction; OR, odds ratio; HIV, human immunodeficiency virus

Introduction

Human papillomaviruses (HPV) are a large and heterogeneous group of viruses of the *Papillomaviridae* family, which are classified into five genera. Currently, more than 200 HPV types have been identified [1]. A subset of mucosal alpha-genus papillomaviruses (alpha-PV) classified as high-risk human papillomaviruses (HR-HPV) cause almost all cervical and a part of vaginal, vulvar, penile, anal, and oropharyngeal cancers. Low-risk alpha-PVs (LR-HPV) are involved in anogenital condyloma in men and women as well as in laryngeal papillomatosis [2]. HPV infection of the skin, usually with the beta-genus papillomaviruses (beta-PV), is common but is mostly asymptomatic in immunocompetent individuals. In 2009, the International Agency for Research on Cancer (IARC) classified two beta-PVs, HPV5 and HPV8, as possibly carcinogenic in *epidermodysplasia verruciformis* patients, who have a high susceptibility to beta-PV infection and skin cancer [1–5].

Head and neck cancer (HNC) is a diverse group of malignancies encompassing cancers of the upper aerodigestive tract, paranasal sinuses, and salivary glands [6]. The most common histological type is squamous cell carcinoma (SCC). HR-HPVs are implicated in the development of a subset of the HNCs [7], most notably in the tonsils. According to a recent comprehensive review, in oral samples from healthy individuals, alpha-PVs can be found in approximately 4.5% of cases [8]; however, other studies have shown slightly greater prevalence rates of 5-12% [9- 12]. There are also studies showing higher prevalence of alpha-PVs in potentially premalignant oral lesions [12, 13].

Since the initial discovery that certain HNCs could be associated with HPV [14], the involvement of HR-HPVs in HNC has been extensively studied [15–17]. However, HPVs of beta- and gamma-genera (cutaneous types) have rarely been the focus of such studies [18–20]. Cutaneous HPVs have been frequently detected in oral cavity specimens [19, 20], but the

prevalence of cutaneous types in cancer tissues has been inconsistent [18, 21]. Those studies mostly used the established FAP59/FAP64 primers amplifying the L1 region of 67 different types [22] or CP65/CP70 and CP66/CP69 nested system amplifying the L1 region of at least 19 HPVs [23].

Herein, the frequency of different beta-PVs in samples from the oral cavity and oropharyngeal cancer patients as well as from clinically distinct oral lesions and gender- and age-matched healthy controls is assessed by a highly sensitive, type-specific, bead-based, multiplex genotyping assay (TS-MPG), which is suitable for the detection of low-copy number HPV cutaneous types and multiple infections in epidemiological analyses [24–27].

Materials and methods

Study population

Samples from patients with primary SCC of the oral cavity (n=35) or oropharynx (n=35), collected [28] from 2001 to 2007 in the Department of Otolaryngology and Head and Neck Surgery, First Faculty of Medicine, Charles University in Prague and University Hospital Motol, with signed informed consent forms, were selected for analysis. For each patient, a control oral rinse sample from a gender- and age-matched healthy individual was collected (n=70). In addition, oral swab samples from 102 individuals with clinically distinct oral lesions (potentially malignant lesions: 40.2% with *leukoplakia*, 30.4% with *lichen ruber planus*, and 2.9% other lesions including two cases of *erythroleukoplakia* and one case of *erythroplakia*; benign proliferative lesions: 10.8% papilloma and 18.6% other lesions including *stomatitis simplex*, *verruca*, *chelitis*, and *aphtae*), collected during dental examination at the Department of Oral Medicine, School of Dental Medicine, University of

Zagreb from 1995 to 2011 [12], were included in this study. The study received the official institutional and ethical approval from the participating institutions.

Patient samples

From oral/oropharyngeal cancer patients, both oral exfoliated cells (lavage) and tumor biopsy specimens were collected as described in detail previously [28]. The corresponding controls provided only lavages. DNA was extracted from a minimum of 250,000 cells using Puregene Core Kit A (Gentra Qiagen, Hilden, Germany) and from two 20 μm formalin fixed paraffin embedded (FFPE) biopsy sections using the Ambion RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Tissues (Applied Bioscience, Austin, TX). A cytobrush (Medscand AB, Sweden) was used for scraping the site(s) of clinically visible lesions, which were classified either by morphology or clinical diagnosis [12] and further confirmed by histopathology [29]. DNA from the collected samples in TES buffer was extracted by the high salt method [12].

Beta-PV detection by Luminex

All samples were analyzed for the presence of beta-PV types using the TS-MPG assay (IARC, Lyon, France), which combines multiplex PCR with a bead-based Luminex technology [24, 30]. This assay detects 43 different beta-PV types, with the human beta-globin gene serving as a control. Following PCR, 10 μl of each reaction mixture was analyzed using the Luminex instrument (Luminex Corporation, Austin, TX) as described previously [25,31].

Data analysis

Samples with multiple infection are those with two or more HPV types detected. Such samples were counted as positive for each type of HPV found. The type-specific HPV prevalence is expressed as the percentage of all subjects tested for HPV, and thus represents the HPV prevalence in both single and multiple infections. The odds ratio (OR) and chi-square test values were calculated in Microsoft Excel (v. 2013).

Results

In this study, 242 patients and controls were tested for beta-PV presence. Overall 312 samples were analysed. Oral cancer patients (median age 55 years, range 35-84) were mostly male (80%), as were the oropharyngeal cancer patients (68.6%, median age 57 years, range 35-75 years). Only 32.4% of patients with oral lesions (median age 55 years, range 16-87) were male.

Thirteen samples in which the beta-globin and HPV amplification was unsuccessful were removed from further calculations. Those were six oral cancer samples, a lavage from one oral cancer patient, three lavages from oropharyngeal controls, and three swabs of oral lesions.

The overall beta-PV positivity is summarized in Figure 1. Table 1 shows detailed comparison of the prevalence of beta-PVs in different groups of patients, matched control group, and different types of samples from the same patient. The oral lesions group contained the most of beta-PV positive samples (67.7%), with the greatest average number of HPV types (5.1) per positive sample, followed by the oral cancer group (55.2%) and oropharyngeal tumor group (45.7%). The lowest positivity rate was found in the lavage samples from patients with oral cancer (17.6%), with the lowest average number of HPV types (1.2) per sample. In contrast,

the oral lavage samples from patients with oropharyngeal cancer were very often positive (60%). The prevalence rate of beta-PV types in the control group was also very high (56.7%).

The beta-PV positivity per patient was evaluated by combining the data from lavage and/or tissue samples of the same patient (Table 1; “Person” column). If either his/her lavage or tissue sample was positive for a beta-PV type, the study subject was considered as positive for that particular beta PV-type. Overall, the beta-PV type most commonly detected in study subjects was HPV5 (35/236; 14.8%), but it was only the third most common one in oral cancer (11.4%) after HPV122 (25.7%) and HPV151 (17.1%).

Table 1. Beta-PV types detected in different study patient groups and gender- and age-matched controls.

	Control (n=67)	Oral cancer			Oropharyngeal cancer			Oral lesion (n=99)
		Lavage (n=34)	Tissue (n=29)	Person** (n=35)	Lavage (n=35)	Tissue (n=35)	Person** (n=35)	
HPV type*	**N [%]**	**N [%]**	**N [%]**	**N [%]**	**N [%]**	**N [%]**	**N [%]**	**N [%]**
Positive	**38 (56.7)**	**6 (17.6)**	**16 (55.2)**	**22 (62.9)**	**21 (60.0)**	**16 (45.7)**	**29 (82.9)**	**67 (67.7)**
Single	**16 (23.9)**	**5 (14.7)**	**5 (17.2)**	**8 (22.9)**	**7 (20.0)**	**8 (22.9)**	**7 (20.0)**	**16 (16.2)**
Multiple	**22 (32.8)**	**1 (2.9)**	**11 (37.9)**	**14 (40.0)**	**14 (40.0)**	**8 (22.9)**	**22 (62.9)**	**51 (51.5)**
HPV5	6 (9.0)	0 (0.0)	4 (13.8)	4 (11.4)	6 (17.1)	5 (14.3)	11 (31.4)	14 (14.1)
HPV8	7 (10.4)	0 (0.0)	1 (3.4)	1 (2.9)	4 (11.4)	4 (11.4)	7 (20.0)	7 (7.1)
HPV9	0 (0.0)	0 (0.0)	1 (3.4)	1 (2.9)	1 (2.9)	1 (2.9)	2 (5.7)	9 (9.1)
HPV12	4 (6.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (8.6)	0 (0.0)	3 (8.6)	9 (9.1)
HPV14	1 (1.5)	0 (0.0)	1 (3.4)	1 (2.9)	2 (5.7)	1 (2.9)	3 (8.6)	2 (2.0)
HPV15	0 (0.0)	1 (2.9)	0 (0.0)	1 (2.9)	2 (5.7)	1 (2.9)	3 (8.6)	5 (5.1)
HPV17	2 (3.0)	1 (2.9)	0 (0.0)	1 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)	7 (7.1)
HPV19	3 (4.5)	2 (2.7)	0 (0.0)	2 (5.7)	0 (0.0)	0 (0.0)	0 (0.0)	9 (9.1)
HPV20	4 (6.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	6 (6.1)
HPV21	2 (3.0)	1 (2.9)	1 (3.4)	2 (5.7)	2 (5.7)	0 (0.0)	2 (5.7)	12 (12.1)
HPV22	3 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)	4 (11.4)	2 (5.7)	6 (17.1)	12 (12.1)
HPV23	2 (3.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (5.7)	0 (0.0)	2 (5.7)	10 (10.1)
HPV24	7 (10.4)	0 (0.0)	0 (0.0)	0 (0.0)	3 (8.6)	2 (5.7)	5 (14.3)	17 (17.2)

HPV25	1 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (3.0)
HPV36	5 (7.5)	1 (2.9)	0 (0.0)	1 (2.9)	1 (2.9)	0 (0.0)	1 (2.9)	18 (18.2)
HPV37	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.0)
HPV38	8 (11.9)	0 (0.0)	0 (0.0)	0 (0.0)	5 (14.3)	0 (0.0)	5 (14.3)	18 (18.2)
HPV47	2 (3.0)	0 (0.0)	3 (10.3)	3 (8.6)	4 (11.4)	4 (11.4)	8 (22.9)	8 (8.1)
HPV49	4 (6.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.9)	1 (2.9)	2 (5.7)	15 (15.2)
HPV75	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.0)
HPV76	6 (9.0)	0 (0.0)	4 (13.8)	4 (11.4)	3 (8.6)	2 (5.7)	4 (11.4)	10 (10.1)
HPV80	3 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (5.7)	0 (0.0)	2 (5.7)	9 (9.1)
HPV92	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)
HPV93	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.9)	0 (0.0)	1 (2.9)	3 (3.0)
HPV96	4 (6.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (3.0)
HPV98	2 (3.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (4.0)
HPV99	1 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	10 (10.1)
HPV100	5 (7.5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.9)	1 (2.9)	2 (5.7)	1 (1.0)
HPV104	1 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (5.7)	2 (5.7)	2 (2.0)
HPV105	3 (4.5)	0 (0.0)	2 (6.9)	2 (5.7)	4 (11.4)	2 (5.7)	5 (14.3)	5 (5.1)
HPV107	3 (4.5)	1 (2.9)	4 (13.8)	4 (11.4)	3 (8.6)	1 (2.9)	4 (11.4)	13 (13.1)
HPV110	8 (11.9)	0 (0.0)	0 (0.0)	0 (0.0)	2 (5.7)	0 (0.0)	2 (5.7)	12 (12.1)
HPV111	3 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (5.7)	3 (8.6)	4 (11.4)	12 (12.1)
HPV113	0 (0.0)	0 (0.0)	1 (3.4)	1 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)	5 (5.1)
HPV115	1 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.0)

HPV118	1 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
HPV120	3 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)	3 (8.6)	2 (5.7)	5 (14.3)	14 (14.1)
HPV122	2 (3.0)	0 (0.0)	9 (31.0)	9 (25.7)	4 (11.4)	5 (14.3)	9 (25.7)	9 (9.1)
HPV124	1 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (5.7)	2 (5.7)	4 (11.4)	11 (11.1)
HPV143	2 (3.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (4.0)
HPV145	5 (7.5)	0 (0.0)	1 (3.4)	1 (2.9)	1 (2.9)	3 (8.6)	3 (8.6)	9 (9.1)
HPV151	2 (3.0)	0 (0.0)	6 (20.7)	6 (17.1)	1 (2.9)	3 (8.6)	3 (8.6)	14 (14.1)

*A particular type was counted in each sample (single or multiple infection) positive for that type (maximum attribution) and thus the sum of detected types exceeds the total of the samples tested.

**Lavage and tissue results were aggregated per person tested.

When the cancer samples were compared to their respective matched controls, the only statistically significant differences for the oral cancer subset were in the prevalence of HPV122 (P=0.026; OR=5.71) and HPV24 (P=0.012; OR=0.06), while in the oropharyngeal cancer subset differed significantly in the prevalence of HPV122 (P=0.002; OR=25.45), HPV5 (P=0.003; OR=15.58), and HPV47 (P=0.003, OR=21.95).

When all cancer samples were compared with all other non-cancer samples (control and oral non-cancer lesions), statistically significant differences were observed in the prevalence of more HPV types: HPV122 (P<0.001; OR=4.97), HPV36 (P=0.011; OR=0.19), HPV47 (P=0.022; OR=2.96), HPV110 (P=0.028; OR=0.22), HPV20 (P=0.037; OR=0.11), HPV99 (P=0.037; OR=0.10), and HPV49 (P=0.044; OR=0.23). However, there were also significant differences in HPV type specific positivity between the subjects with oral lesions and healthy controls: HPV9 (P=0.011; OR=14.8), HPV151 (P=0.015; OR=5.6), HPV124 (P=0.016; OR=8.63), HPV99 (P=0.028; OR=7.75), HPV120 (P=0.04; OR=3.68), HPV36 (P=0.043; OR=2.89), and HPV21 (P=0.045; OR=4.69).

Another result of particular interest was a low concordance between the typing results of biopsy tissue and oral rinse samples from the same cancer patient (n=70). Overall, there were seven (10%) patients who had one of the two samples beta-globin negative and were excluded from analysis, 32 (45.7%) patients with HPV detected in only one sample and the other sample being HPV negative, 18 (25.7%) patients whose samples were both HPV negative, , and finally 13 (18.6%) patients who tested positive for HPV in both types of material. The low concordance of HPV types in the 13 patients with both samples positive for at least one HPV type is illustrated in Table 2.

Table 2. HPV typing concordance between different samples from the same patient (n=13).

Sample		HPV100	HPV104	HPV105	HPV107	HPV110	HPV111	HPV12	HPV120	HPV122	HPV124	HPV14	HPV145	HPV15	HPV151	HPV19	HPV21	HPV22	HPV23	HPV24	HPV38	HPV47	HPV49	HPV5	HPV76	HPV8	HPV80	HPV9	HPV93
8	**OPH L**			1	1		1		1					1					1						1				
	OPH T			1			1													1									
10	**OPH L**													1				1						1		1			
	OPH T									1																1			
13	**OPH L**									1																	1		1
	OPH T								1																				
23	**OPH L**							1																					
	OPH T																	1											
30	**OPH L**					1																							
	OPH T											1								1			1			1			
62	**OPH L**																	1						1					
	OPH T																									1			
79	**OPH L**																						1						
	OPH T																											1	
87	**OPH L**			1	1				1			1															1		
	OPH T						1			1			1		1														
110	**OPH L**							1					1		1		1					1				1			
	OPH T	1					1			1	1		1		1									1					
122	**O L**															1													
	O T									1					1														
167	**O L**				1																								
	O T				1					1					1														
178	**OPH L**										1									1	1				1				
	OPH T		1		1					1								1						1	1				
184	**OPH L**																					1							
	OPH T																							1					

OPH = oropharyngeal cancer; O = oral cancer; L = lavage fluid, T = tissue

Discussion

The alpha-PVs are a well acknowledged etiological factor in the development of some types of head and neck cancers. However, data on the potential influence of beta-PVs are limited. The purpose of this study was to provide additional data on the beta-PV prevalence in oral and oropharyngeal cancers, potentially premalignant lesions, and corresponding controls. To this end, 35 oral and 35 oropharyngeal cancer cases, 102 patients with pre- and non-malignant oral mucosa lesions, and 70 healthy controls were evaluated by a highly sensitive TS-MPG assay for beta-PV genotyping.

In this study, HPV was found in 41.8%, 54.9%, and 67.7% of samples from the oral region of cancer patients and controls, oropharyngeal region of cancer patients and controls, and oral region of oral lesion patients, respectively. The high average beta-PV prevalence observed in this study in any group investigated is difficult to put in context with previous studies. Koskinen et al. [21] tested 61 HNC tissues with a single-phase FAP59/64 PCR and nested PCR with primers CP65/70 and CP66/69 to detect cutaneous HPVs but failed to find any. Lindel et al. [18] have reported HPV in 35.0% of 51 HNC tissues where, aside from HPV6 and HPV16, most of the detected types belonged to the cutaneous types. Bottalico et al. [19] investigated oral rinse samples from 317 HIV negative and otherwise healthy men by FAP59/64 primers and hybridization and have found a beta-PV prevalence of 34.0% (108/317). Paolini et al. [20] tested oral rinse/swab samples from healthy controls, non-malignant lesions, and biopsy material from HNC. They used CP65/CP70 followed by CP66/CP69 and FAP59/FAP64 primers. The healthy group had 25.0% beta-PV positive samples, the patients with lesions had the highest beta-PV prevalence (51.0%), and only 20.5% of cancer tissues were beta-PV positive. The authors noted the methodological differences between their study and all three previous ones and also emphasized that beta-PVs

are usually present in a very low copy number. Therefore, the higher prevalence of beta-PVs in our study can be attributed to using a more recent and sensitive genotyping method. It should also be noted that their control group had an average age of 40.7 years, while our controls were age matched to the cancer patients and thus had an average age of 56.2 years, which might have influenced the results [10].

The overall most common type in the analyzed samples was HPV5 (11.7%). This HPV type was found in 9.0% samples from the combined control group, of which 11.4% and 31.4% were detected in oral and oropharyngeal cancer samples, respectively. This HPV type is grouped as probably carcinogenic and associated with non-melanoma skin cancer [4] but was also the most common beta-PV type to be found in the oral cavity of healthy people [19]. Herein, there was a large statistically significant difference between the oropharyngeal cancer patients and matched controls (P=0.003; OR=15.58), indicating that HPV5 should be studied more closely.

The second leading beta-PV type in this study was HPV38 (10.4%). It is known to be highly prevalent in skin cancer [32, 33] but is also very common in the plucked eyebrow hairs of healthy people [34]. Antibodies against this type are also common in the general population [35]. Strangely, even though HPV38 was often identified in oral cancer in a previous study [36], none of the oral cancer patients had this type detectable in either lavage or biopsy sample in our study. However, comparable rates of the oropharyngeal cancer patients (14.3%) and controls (11.9%) had this type present in the present study.

The third overall most common beta-PV type was HPV122 (9.7% of samples tested; 12.3% of individuals tested). Despite the data on this type being scarce, in this study, HPV122 was found to be statistically significantly associated with both oral (P=0.026; OR=5.7) and

oropharyngeal (P=0.002; OR=25.5) cancer. Furthermore, the association was also significant in the overall comparison of cancer *versus* all non-cancer samples (including control and non-cancer oral lesions). The lack of other reports of the presence of this HPV type in HNC might be due to the fact that it was only recently described [1], and some of the methods previously used for the detection of beta-PVs do not specifically target HPV122.

The prevalence of alpha-PVs in the same cancer patients has been previously determined, and as expected, alpha-PVs were more often found in the cancer patients (49.0%) compared with the matched controls (12.0%) [11]. However, a study on a subset of the same cancer patients comparing tumor tissue and lavage specimens from the same patient has observed that the alpha-PV overall positivity rate is slightly lower in the rinse specimens than in tumor tissue specimens (53.0% vs 59.0%), with a greater diversity of types and multiple infection being more often found in the rinse specimens (12.0% vs 2.4%) [28]. The difference in alpha-PV positivity of oral cancer patients between tumor tissue and rinse specimens was more pronounced (16.7% vs 8.3%) in comparison with oropharyngeal cancer patients (67.5% vs 62.4%). Similarly, more than double difference was found in beta-PV positivity of oral cancer patients between cancer tissue and rinse samples (55.2% vs 17.7%). On the other hand, in oropharyngeal cancer patients, unlike the alpha-PVs, the beta-PVs were less prevalent in tissue samples (45.7%) than in the rinse samples (60%). The co-positivity rates for alpha- and beta-types were 1%, 6%, 10%, and 29% in controls, oral cancers, oral lesions, and oropharyngeal cancers, respectively, but with no correlation (correlation coefficients ranged from -0.2 to 0.09, without statistical significance) (Table 3).

Table 3. Positivity of controls and patients with oral/oropharyngeal tumors for HPVs of alpha and beta-genera.

			Alpha-HPV N [%]*		Overall HPV prevalence [%]	
	Group of subjects	N	Positive	Negative	Alpha	Beta
Beta-HPV N [%]	**Controls**	**67**	**5**	**62**	7.5	56.7
	Positive	38	1 (1.5)	37 (55.2)		
	Negative	29	4 (6.0)	25 (37.3)		
	Oral lesions	**99**	**14**	**85**	14.1	67.7
	Positive	67	10 (10.1)	57 (57.6)		
	Negative	32	4 (4.0)	28 (28.3)		
	Oral cancers	**63**	**9**	**54**	14.3	34.9
	Positive	22	4 (6.3)	18 (28.6)		
	Negative	41	5 (7.9)	36 (57.1)		
	Oropharyngeal cancers	**70**	**41**	**29**	58.6	52.9
	Positive	37	20 (28.6)	17 (24.3)		
	Negative	33	21 (30.0)	12 (17.1)		

*percentages were calculated on the sample group subtotal

One of the limitations of the present study is the availability of different types of samples collected from cancer patients and the respective controls. However, prior studies often used the same types of clinical material [21] due to ethical and medical reasons preventing biopsy sampling of healthy subjects. Thus, our data are readily comparable to those from the previous studies in this regard. Furthermore, in our present study, we were able to compare the prevalence and type-specificity in two different types of the clinical material taken from the same patient. We have previously shown for Alpha PV types that while in tumor tissue, only HR HPV types are present and multiple infection is rare, in oral rinses, multiple infection is more frequent and also LR HPV types as a single infection can be detected. Nevertheless, in patients with HR HPV-positive tumors, a concordant HR HPV type was present in 97.0% of oral rinses, suggesting viral shedding from the tumor [29]. In contrast, the concordance was poor for Beta-PV types. We hypothesize that it is due to the non-etiological association of the Beta-PV types with studied type of cancers. It is important to note that the biggest difference in the two types of materials analyzed in cancer patients is that the oral rinse provides information about the presence of viruses in the whole oral and proximal part of the oropharyngeal area while tissue sections provide information of the presence of HPV types in the tumor itself and in its direct proximity.

One of the major strengths of this study is the use of the optimized multiplex PCR/Luminex assay that can readily distinguish a large number of genotypes in a single sample. The previous methods based on sequencing of PCR amplicons generated by consensus primers were limited in this regard.

In summary, this study assessed the presence of the so far understudied beta-PVs in HNC patients, matched controls, and subjects with oral lesions using the newest TS-MPG assay specifically designed to target beta-PVs. The most prevalent type was the probably

carcinogenic HPV5, while the second most common was HPV38. The third leading type was HPV122 that has been so far almost completely absent from the literature, and herein it was found to be statistically significantly associated with cancer, which might warrant further interest and study. Finally, poor HPV type concordance between patient matched lavage and tissue samples suggests that beta-PVs are not strongly etiologically linked to HNC, even if some associations are statistically significant.

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Competing interests

The authors have no competing interests.

Ethical approval

All study participants signed an informed consent form. The study received the official institutional and ethical approval from the participating institutions according to the national regulations.

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Figure 1. Beta-PVs detected in head and neck squamous cell carcinomas (HNC) and oral lesions (the control groups are age and sex matched to HNC patients).