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Low human papillomavirus prevalence in head and neck cancer: results from two large case–control studies in high-incidence regions

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Background Recent studies support an important role for human papillomavirus (HPV) in a subgroup of head and neck squamous cell carcinomas (HNSCC). We have evaluated the HPV deoxyribonucleic acid (DNA) prevalence as well as the association between serological response to HPV infection and HNSCC in two distinct populations from Central Europe (CE) and Latin America (LA).

Methods Cases ($n = 2214$) and controls ($n = 3319$) were recruited from 1998 to 2003, using a similar protocol including questionnaire and blood sample collection. Tumour DNA from 196 fresh tissue biopsies was analysed for multiple HPV types followed by an HPV type-specific polymerase chain reaction (PCR) protocol towards the E7 gene from HPV 16. Using multiplex serology, serum samples were analysed for antibodies to 17 HPV types. Statistical analysis included the estimation of adjusted odds ratios (ORs) and the respective 95% confidence intervals (CIs).

Results HPV16 E7 DNA prevalence among cases was 3.1% (6/196), including 4.4% in the oropharynx (3/68), 3.8% in the hypopharynx/larynx (3/78) and 0% among 50 cases of oral cavity carcinomas. Positivity for both HPV16 E6 and E7 antibodies was associated with a very high risk of oropharyngeal cancer (OR = 179, 95% CI 35.8–899) and hypopharyngeal/laryngeal cancer (OR = 14.9, 95% CI 2.92–76.1).

Conclusions A very low prevalence of HPV DNA and serum antibodies was observed among cases in both CE and LA. The proportion of head and neck cancer caused by HPV may vary substantially between different geographical regions and studies that are designed to evaluate the impact of HPV vaccination on HNSCC need to consider this heterogeneity.

Keywords Head and neck cancer, human papillomavirus (HPV), Central Europe, Latin America

Introduction

Approximately 570 000 new cases of head and neck squamous cell carcinoma (HNSCC) are diagnosed annually worldwide, representing 5% of all tumours.[1] Smoking and alcohol drinking are the major risk factors. Early case studies suggested that human papilloma virus (HPV) infection may play a causal role in a subgroup of predominantly oropharyngeal, and especially tonsillar, HNSCC.[2–7] These findings were further confirmed by larger epidemiological studies.[8–15] About 95% of HPV-associated HNSCC harbour DNA of HPV type 16.[16] Latin America (LA) and Central Europe (CE) are relatively high-risk regions for HNSCC, with incidence rates of 15 and 3.1/100 000 for males and females in LA, and 23.2 and 2.6 for men and women in CE, respectively.[1]

Two prophylactic HPV vaccines against HPV 6, 11, 16 and 18 (quadrivalent) or HPV16 and 18 (bivalent) have been developed, and studies have shown very high efficacy to prevent infection and HPV-related diseases.[17,18] However, until now, phases II and III clinical trials focused only on genital infection or cervical intraepithelial neoplasia grade 2 or worse (CIN 2+) as endpoints, and no efficacy or cost–benefit studies focussing on non-genital cancers have been conducted.[19]

The reported prevalence of HPV DNA in HNSCC ranges widely[13,20,21] with this disparity being related to differences in the anatomical site of the tumours as well as distinct methods for HPV detection.[13] Serum HPV antibodies are mostly type specific[22] and present a good correlation with the presence of viral DNA in the tumour.[23] Serology for HPV16 L1 has been proposed as a surrogate marker of cumulative HPV exposure, thus allowing the investigation of the association between infection and cancer.[22] Antibodies to the HPV16 E6 and E7 oncoproteins are generally viewed as markers of invasive HPV-related tumours, but only a few studies have evaluated the association between HNSCC and HPV focussing on serological markers of these oncoproteins.[12,24–29] In a previous International Agency for Research on Cancer (IARC) multicentre study,[12] antibodies to HPV16 E6 or E7 were associated with an increased risk for both oral [odds ratio (OR) = 2.9; 95% confidence interval (CI) 1.7–4.8] and oropharyngeal cancer (OR = 9.2; 95% CI 4.8–17.7). The prevalence of HPV16 E6 or E7 antibodies was 4.6% for oral and 11.9% for oropharyngeal cancers among the 1546 cases (243 oropharynx).[12] Recently, Smith *et al*.[27] have reported that, among 204 cases and 326 controls from a US population, the presence of antibodies to HPV16 E6 (OR = 32.8; 95% CI 9.7–110.8) or HPV16 E7 (OR = 37.5; 95% CI 8.7–161.2) conferred a very high risk for head and neck cancer. The prevalence of these antibodies among the cases was 20.6% and 18.6%, respectively. Furthermore, D'Souza *et al*.[8] described that, among 100 cases and 200 controls from an outpatient otolaryngology clinic of the Johns Hopkins Hospital in Baltimore, antibodies to HPV16 E6 and/or E7 (OR = 58.4; 95% CI 24.2–138.3) were associated with a high risk for oropharyngeal cancer. Again, a high prevalence was found among cases, with 64% for either/both HPV16 E6/E7.

The attributable risk of HPV16 E6 or E7 for oropharyngeal cancer has been estimated to be >50% in both of the recent US studies.[8,27] In this study, the main objective was to examine the importance of the association between HPV infection and the risk of HNSCC in two high-risk populations from LA and CE using a common protocol for data and specimen collection, and one central laboratory for HPV evaluation.

Methods

Participants

Two hospital-based case–control studies were conducted in CE and LA to assess lifestyle, occupational and genetic risk factors for head and neck cancers. Cases and controls were recruited in Russia (Moscow), Romania (Bucharest), Poland (Lodz and Warsaw), Czech Republic (Olomouc and Prague) and Slovakia (Banská Bystrica) from 1998 to 2002 and in Argentina (Buenos Aires), Cuba (La Habana) and Brazil (Rio de Janeiro, São Paulo, Pelotas, Porto Alegre and Goiânia) from 1998 to 2003. Both studies were coordinated by the IARC according to a similar protocol for both case and control recruitment. Incident cancer cases included patients with cancer of the oral cavity, oropharynx, hypopharynx/pharynx and larynx. A total of 2905 cases were recruited at participating hospitals with blood samples

collected and valid information on HPV serology for 2214 cases (CE = 544 and LA = 1670).

Trained medical staff reviewed medical records to extract relevant diagnostic information, including date and method of diagnosis, histological type, tumour location and stage. All cases had a histologically confirmed diagnosis of squamous cell carcinoma and were enrolled consecutively. Staging was based on the TNM Classification—5th edition.

Eligible controls included residents of the study areas for at least 1 year, who were admitted to the same hospitals as the cases or in a comparable catchment area (population-based controls were enrolled in Warsaw). Only controls presenting conditions unrelated to tobacco use or alcohol consumption were included. Recruitment of controls took place at the same time as the cases and, within each centre, no single diagnostic category made up >20% of the overall control group. Response rates were high in all centres, with a minimum response rate of 75%.

Trained interviewers conducted in-person interviews to elicit information on demographical background, tobacco use and alcohol consumption, occupational history and dietary and other lifestyle habits using identical protocols across all centres for each study.[30] Each centre received approval by an ethical committee before commencing the study.

Biological material

Blood was obtained at the time of the interview for cases and controls from both studies. Oral cells or biopsy samples were obtained only for the LA study.

Oral cells from controls were sampled by performing superficial scrapes of the oral mucosa with a tooth brush, followed by a mouthwash with phosphate buffered saline (PBS) that included gargles for throat sampling. Valid samples were obtained for 898 of 1288 individuals (69.7%).

Patients with head and neck tumours provided oral cells, and a biopsy sample was obtained from a limited number of cases from Argentina and Brazil, normally during surgery. Oral cells were collected from 1054 of 1670 cases (63.1%) and frozen tumour tissue from 507 of 1670 cases (30.4%).

Detection of HPV antibodies

Serum samples were analysed using multiplex serology, an antibody detection method based on a glutathione *S*-transferase capture ELISA, at the German Cancer Research Center (DKFZ) as previously described,[31,32] in combination with fluorescent bead technology.[33,34]

Mean fluorescence intensity (MFI) values were dichotomized as antibody positive or negative. Standardized cut-offs for HPV L1 antibodies (except HPV6 L1) were defined earlier and applied here.[35,36] Seropositivity cut-offs for HPV6 L1 and HPV early (E) proteins were determined using a similar algorithm for serum samples of 117 females, HPV DNA-negative, self-reported virgins from a cross-sectional study among Korean students.[35] These sera were analysed side-by-side with the CE and LA serum samples. The mean +5 SDs were calculated (except for HPV6 L1, for which mean +3 SDs excluding the remaining positive outliers were used), and values below 100 MFI were set to this minimum cut-off point. The resulting single cut-off was doubled to stringently separate sero-positive and -negative reactions.[12]

DNA extraction

Fresh tumour tissue from cases l was frozen and then transported to a central laboratory in São Paulo, Brazil, where DNA was extracted using a commercial kit (QIAamp DNA Mini Kit, Qiagen, Valencia, CA, USA). Total DNA was eluted in 140 µl of ddH_2O and quantified by reading in a spectrophotometer at 260 nm. DNA was diluted to 50 ng/µl, and 5 µl (250 ng) were applied in a polymerase chain reaction (PCR). If the original DNA concentration was <50 ng/µl, 5 µl of the eluate was used directly. Oral cells were kept under refrigeration (2–8°C) up to 72 h before they were centrifuged at 3000*g* for 10 min at room temperature. The cell pellet was resuspended in proteinase K digestion buffer and processed as detailed above.

PGMY09/11 PCR

DNA was submitted to PCR with primers PGMY09/11 spanning ~450 bp from the L1 region of most HPV types,[37] in the presence of human β-globin primers, amplifying a fragment of 268 bp, for quality control. Reaction conditions were 200 µM of dNTPs, 4 mM of magnesium chloride, 80 nM of PGMY09/11 and 20 nM of PCO4/GH20 (β-globin) oligonucleotides, 250 ng of template DNA and 1 U of Taq polymerase (Invitrogen, São Paulo, Brazil). Thermocycling profile consisted of an initial incubation of 5 min at 94°C plus 40 cycles of 94°C (1 min), 55°C (1 min), 72°C (1 min) and a final elongation step of 5 min at 72°C, in a PE 2400 (Applied Biosystems, Foster City, CA, USA) or MasterCycler gradient thermal cycler (Eppendorf AG, Hamburg, Germany). PCR products were analysed by electrophoresis on a 2% agarose gel stained with ethidium bromide and observed under ultraviolet (UV) light.

For confirmation of initially positive results, samples displaying a band of 450 bp were submitted to the same PCR protocol described above but after omitting the β-globin primers. If confirmed positive, they were typed by restriction fragment length polymorphism (RFLP) using the enzymes and patterns described by Bernard *et al*.[38]

Negative controls comprised HPV-negative human leucocyte DNA and a tube with water, instead of DNA, at the PCR set-up. Positive controls consisted of an HPV-positive cervical sample with an unknown amount of HPV 16 DNA and the human leucocyte

DNA spiked with 10 pg of HPV16 plasmid per 250 ng of human DNA. The detection limit of this method was shown to be 1 pg of HPV DNA in a background of 250 ng of human DNA at the 99% hit rate. This analysis was conducted at the Tropical Medicine Institute, University of São Paulo, Brazil.

HPV 16 E7 PCR

A sub-series of 196 selected tumours was also submitted to an HPV type-specific PCR protocol towards the E7 gene from HPV16, in order to corroborate the findings with the above-described generic primer PCR (PGMY) targeting the L1 gene. This selection was made in order to analyse all tonsillar tumours that had adequate DNA available ($n = 33$), all PGMY09/11 PCR positive tumours ($n = 4$) and also to keep approximately the same gender and tumour-site distribution as present across the whole series (oral cavity, $n = 50$; oropharynx, $n = 68$; hypopharynx/larynx, $n = 78$). A 277-bp fragment of the E7 gene of HPV16 was amplified by PCR using the HPV16 E7-specific primers 5′-CAT GGA GAT ACA CCT ACA TTG-3′ and 5′-CAG ATG GGG CAC ACA ATT CC-3′, spanning nucleotides (nt) 565–842 (*GenBank FJ006723.1*). The reaction mix contained 1× PCR buffer, 200 μM of each dNTP, 0.2 μM of each primer and 0.625 U of HotStar Taq DNA polymerase (QIAGEN, Hilden, Germany) in a final volume of 25 μl. An initial 15-min denaturation step at 95°C was followed by 40 cycles of amplification in a GeneAmp PCR system 2700 thermocycler (PE Applied Biosystems, Foster City, CA, USA). Each cycle included a denaturation step at 94°C for 1 min, an annealing step at 55°C for 1 min and an elongation step at 72°C for 1 min. The final elongation step was prolonged for further 10 min. Amplicons were visualized on 2% agarose gels stained with ethidium bromide. The analytical sensitivity of this PCR was investigated using 10 000–0 copies of HPV16 viral genomes as templates, and a PCR product was obtained with 100 copies. This step of the analysis was conducted at the IARC, Lyon, France.

Covariates

Covariates included age, gender, country, tobacco smoking and alcohol drinking. Lifetime smoking habit was measured in tobacco pack-years, and subjects were also classified as ever/never-smokers. A tobacco pack-year was defined as smoking the equivalent of one pack of cigarettes (or the equivalent amount of cigar or pipe) daily for 1 year. A former smoker was defined as having quit for at least 2 years, and a never-smoker had not used more than 100 cigarettes or the equivalent of cigars or pipes in his/her lifetime. No data was collected on smokeless tobacco use, as this is very rare in both study populations.

Lifetime alcohol consumption was calculated as the product of the ethanol content (in grams), frequency and duration of consumption for all types of beverage. A never-drinker had never consumed alcoholic drinks (CE) or had consumed them less than once a month (LA).

Statistical analysis

Unconditional logistic regression was used to estimate ORs and the corresponding 95%CIs. Unless otherwise specified, all ORs were adjusted by age, sex, country, tobacco smoking and alcohol drinking. Analyses were performed with Stata software, version 9.0 (StataCorp, College Station, Texas, TX, USA). Effect modification was assessed by stratifying for tobacco smoking (never/former vs current), alcohol drinking (never/former vs ever), age ($\leqslant 50$ or >50 years), sex, number of sexual partners and oral genital contact. Cases and controls with missing data were excluded from the analysis.

In order to test the significance of a multiplicative interaction between HPV16 E6 seropositivity and smoking, we performed the likelihood ratio test by comparing the model with HPV16 E6 and smoking to the model with HPV16 E6 seropositivity, smoking and the interaction term. Population-attributable risk percentages (PAR%) were estimated using the following formula:

$$\frac{\text{Prevalence of exposure among controls} \times (\text{OR} - 1)}{\text{Prevalence of exposure among controls} \times (\text{OR} - 1) + 1}$$

A case-only analysis was used to assess the associations between HPV infection and staging. For all statistical tests, results were considered statistically significant when $P < 0.05$.

Results

There were 2214 cases and 3319 controls with complete serology information (Table 1). The majority of cases were males (86.9%), and cases were significantly more likely than controls to be past or current smokers or drinkers (Table 1). Most tumours were located in the hypopharynx/larynx ($n = 1088$, 49.2%), followed by the oral cavity ($n = 687$, 31.0%) and the oropharynx ($n = 439$, 19.8%). Of the oropharyngeal tumours, 101 were tonsillar.

When the two studies were analysed together, antibodies against HPV16 L1 were not significantly associated with the risk of HNSCC (OR = 1.02, 95% CI 0.80–1.30) or specific tumour sites (Table 2). Similar results were observed when LA and CE studies were analysed separately. The prevalence of antibodies against HPV16 E6 was 2.5% and 0.9% for cases and controls, respectively (OR = 3.82, 95% CI 2.21–6.59), and antibodies against HPV16 E7 were detected in 3.2% of cases and 1.7% of controls (OR = 1.71, 95% CI 1.13–2.58). Double seropositivity for both HPV16 E6 and E7 was found in 22 cases (1.0%) but only in three controls (0.1%), and conferred a high risk for the development of HNSCC (OR = 19.0, 95% CI 5.24–69.0). The analysis by tumour site revealed that the

Table 1 Baseline characteristics of cases and controls and risks associated with smoking and alcohol drinking

Variable	Category	Cases (%) ($n=2214$)	Controls (%) ($n=3319$)	OR[a] (95%CI)
Sex	Male	1923 (86.9)	2468 (74.4)	–
	Female	291 (13.1)	851 (25.6)	
Age (years)	⩽40	68 (3.1)	151 (4.5)	–
	41–50	455 (20.5)	583 (17.6)	
	51–60	774 (35.0)	1078 (32.5)	
	61–70	633 (28.6)	1036 (31.2)	
	71–80	261 (11.8)	440 (13.3)	
	>80	23 (1.0)	31 (0.9)	
Country	Argentina	328 (14.8)	202 (6.1)	–
	Brazil	1263 (57.0)	1043 (31.4)	
	Cuba	79 (3.6)	43 (1.3)	
	Russia	268 (12.1)	694 (20.9)	
	Slovakia/Czech Republic	27 (1.2)	475 (14.3)	
	Romania	79 (3.6)	147 (4.4)	
	Poland	170 (7.7)	715 (21.6)	
Smoking	Never	121 (5.5)	1099 (33.1)	1.00
	Former smoker	424 (19.2)	950 (28.6)	2.64 (2.08–3.37)
	Current	1664 (75.3)	1270 (38.3)	10.1 (8.08–12.7)
Alcohol drinking	Never	154 (6.9)	550 (16.5)	1.00
	Only in the past	701 (31.7)	1220 (36.8)	2.42 (1.89–3.10)
	Current	1359 (61.4)	1549 (46.7)	2.85 (2.23–3.63)
Tumour site	Oral	687 (31.0)	–	–
	Oropharynx	439 (19.8)		
	Hypopharynx/larynx	1088 (49.2)		

[a]ORs adjusted by age, sex, smoking, alcohol drinking and country, where appropriate.

association of antibodies to HPV16 E6 or E7 proteins was strongest with oropharyngeal cancer (OR = 13.2, 95% CI 5.87–29.5 for HPV16 E6; OR = 4.46, 95% CI 2.40–8.26 for HPV16 E7; and OR = 179, 95% CI 35.8–899 for double seropositivity). When the analysis was restricted to tonsillar cancer ($n=101$), the seroprevalence of HPV16 E6 was 10.9% and 0.9% for cases and controls, respectively (OR = 22.0, 95% CI 8.26–58.9, $P<0.001$), and antibodies against HPV16 E7 were detected in 8.9% of cases and 1.7% of controls (OR = 5.74, 95% CI 2.45–13.44). Seroprevalence for both HPV16 E6 and E7 was 8.9% among tonsillar cases ($n=9$), but only 0.1% among controls ($n=3$), corresponding to >300-fold increased risk for the development of tonsillar carcinoma (OR = 331, 95% CI 47.4–2307). In addition, seropositivity for HPV16 E6 (OR = 2.82, 95% CI 1.40–5.66) and HPV16 E6/E7 double seropositivity (OR = 14.9, 95% CI 2.92–76.1) was associated with an increased risk for hypopharyngeal/laryngeal cancer. When results were stratified by site, consistent results were observed for both larynx (HPV16 E6—OR = 2.91, 95% CI 1.43–5.95; both E6/E7—OR = 13.7, 95% CI 2.29–82.3) and hypopharynx (HPV16E6—OR = 2.78, 95% CI 0.58–13.4; both E6/E7—OR = 31.3, 95% CI 2.69–362). Neither HPV16 E6 nor E7 antibodies were associated with cancer of the oral cavity (Table 2).

When the two case–control studies were analysed separately, there was no difference between the LA and CE studies in the HNSCC risk associated with positivity for HPV16 L1 ($P=0.576$) and E7 ($P=0.096$) antibodies. However, the HNSCC risk associated with HPV16 E6 antibodies was >4-fold higher in LA than in CE ($P=0.012$). The same effect was observed for the association between antibodies to HPV16 E6 and oropharyngeal cancer (P for heterogeneity = 0.003; Table 2).

Among controls, seroprevalence for HPV16 L1 in LA was significantly higher than in CE ($P=0.012$). There was no significant difference for either HPV16 E6 ($P=0.213$) or HPV16 E7 seroprevalence ($P=0.222$; Table 2).

Of all analysed HPV L1 proteins from cutaneous types (HPV 1, 4, 8, 38, 49 and 77) or low-risk types

Table 2 HPV16 seroprevalence (L1, E6 and E7 proteins) and risk associated with HNSCC and individual tumour sites

HPV 16 antibody	Study	All HNSCC cases ± (prevalence %)	Controls ± (prevalence %)	OR[a] (95% CI)	Oral cavity		Oropharynx		Hypopharynx/larynx	
					±	OR[a] (95% CI)	±	OR[a] (95% CI)	±	OR[a] (95% CI)
L1	Pooled	172/2042	239/3080	1.02	51/636	0.93	35/404	1.12	86/1002	1.15
		(7.8)	(7.2)	(0.80–1.30)		(0.65–1.32)		(0.73–1.72)		(0.86–1.56)
	LA	139/1531	111/1177	1.06	45/486	1.08	31/329	1.19	63/716	1.11
		(8.3)	(8.6)	(0.79–1.42)		(0.73–1.62)		(0.74–1.93)		(0.77–1.60)
	CE	33/511	128/1903	0.91	6/150	0.47	4/75	0.79	23/286	1.23
		(6.1)	(6.3)	(0.58–1.42)		(0.19–1.14)		(0.27–2.32)		(0.73–2.07)
P-value			0.012	0.576*		0.096*		0.495*		0.752*
E6	Pooled	56/2158	29/3290	**3.82**[3]	11/676	1.92	24/415	**13.2**[3]	21/1067	**2.82**[2]
		(2.5)	(0.9)	**(2.21–6.59)**		(0.83–4.45)		**(5.87–29.5)**		**(1.40–5.66)**
	LA	46/1624	8/1280	**7.90**[3]	8/523	2.18	22/338	**42.9**[3]	16/763	**5.17**[2]
		(2.7)	(0.6)	**(3.38–18.5)**		(0.69–6.86)		**(12.8–144)**		**(1.86–14.4)**
	CE	10/534	21/2010	1.67	3/153	1.75	2/77	1.97	5/304	1.38
		(1.8)	(1.0)	(0.70–3.98)		(0.46–6.61)		(0.38–10.2)		(0.46–4.17)
P-value			0.213	**0.012***		0.807*		**0.003***		0.085*
E7	Pooled	70/2144	58/3261	**1.71**[1]	22/665	1.73	26/413	**4.46**[3]	22/1066	1.04
		(3.2)	(1.7)	**(1.13–2.58)**		(0.96–3.11)		**(2.40–8.26)**		(0.59–1.83)
	LA	61/1609	27/1261	**2.27**[2]	19/512	1.79	25/335	**5.75**[3]	17/762	1.26
		(3.6)	(2.1)	**(1.35–3.81)**		(0.92–23.5)		**(2.88–11.5)**		(0.63–2.52)
	CE	9/535	31/2000	0.98	3/153	2.05	1/78	0.83	5/304	0.80
		(1.6)	(1.5)	(0.42–2.27)		(0.53–7.94)		(0.09–7.01)		(0.28–2.26)
P-value			0.222	0.096*		0.900*		0.097*		0.477*
E6 and E7[b]	Pooled	22/2110	3/3235	**19.0**[3]	1/655	1.82	16/405	**179**[3]	5/1050	**14.9**[3]
		(1.0)	(0.1)	**(5.24–69.0)**		(0.09–37.1)		**(35.8–899)**		**(2.92–76.1)**

[a]OR adjusted by age, sex, smoking, alcohol drinking and country.
[b]Comparison of double HPV16 E6 and E7 positives against double HPV16 E6 and E7 negatives.
**P*-value for heterogeneity.
LA = Latin America; CE = Central Europe.
[1]$p < 0.05$; [2]$p < 0.01$; [3]$p < 0.001$.

(HPV 6 and 11), only antibodies to HPV6 L1 were significantly associated with all HNSCC cases (OR = 1.27, 95% CI 1.09–1.47), oral cancer (OR = 1.43, 95% CI 1.16–1.75) and hypopharyngeal/laryngeal cancer (OR = 1.22, 95% CI 1.02–1.46), but not with oropharyngeal cancer.

In a pooled analysis of both studies, antibodies to E6 or E7 proteins of the low-risk types (HPV 6 and 11) and the high-risk HPV types 18 and 45 were not associated with HNSCC or particular tumour sites. We observed associations of antibodies to E6 and/or E7 proteins of the high-risk types HPV 31, 33, 35, 52 and 58 mainly with oropharyngeal cancer (data not shown). However, all of these HPV types are phylogenetically closely related to HPV16, and the antibody titres among HPV16 E6 or E7 seropositive cases were very high (E6: median 3246 MFI, range 485–20 355 MFI; E7: median 1828 MFI, range 1104–13 371 MFI). After exclusion of subjects that were seropositive for the homologous HPV16 proteins, the only remaining significant association was found for antibodies to HPV52 E6 and oropharyngeal cancer (6 positive out of 415 cases and 7 positive out of 3290 controls—OR = 9.15, 95% CI 1.87–44.8).

According to the effect estimates, double seropositivity to HPV16 E6 and E7 is the most specific marker for HPV-associated HNSCC (Table 2). However, there were too few double seropositives for additional stratification. Therefore, we concentrated our further analyses on antibodies to HPV16 E6, which are less prone to antibody cross-reactivity and also showed the highest concordance with HPV tumour DNA.

The associations between HNSCC (and also for each tumour site) and seropositivity for HPV16 E6, stratified by age, sex, smoking, alcohol drinking, number of sexual partners and orogenital contacts are shown in Table 3. There was a striking difference between age groups regarding the association between HPV16 E6 and oropharyngeal cancer (10-fold increased in the younger group, P for heterogeneity = 0.017). Similar effects, but less strong, were also seen for the hypopharynx/larynx (>2-fold, $P = 0.356$), and all HNSCC (4.5-fold, $P = 0.020$). In addition, the association between HPV16 E6 and oropharyngeal cancer was 7-fold higher among never-smokers compared with former or current smokers (P for heterogeneity = 0.022). It is important to point out that six out of nine (66.7%) never-smokers with oropharyngeal cancer were HPV16 E6 seropositive. Data on the number of sexual partners and oral genital contacts were only available from the LA study, and there was a 10-fold increase in risk for HNSCC and cancer of the oropharynx and the hypopharynx/larynx for those with 11 or more reported sexual partners compared with $\leqslant 10$. However, no effect modification by sex, alcohol drinking or orogenital contact was observed (Table 3).

We analysed the effect of smoking on the association of HPV16 E6 serology with HNSCC in detail and found no effect for all tumour sites combined (Figure 1). For oropharyngeal cancer however, a significant interaction ($P = 0.02$), less than multiplicative, was found.

The HPV16 L1 DNA prevalence in 507 tumour tissue samples (by PGMY09/11 PCR) was 0.6% (95% CI 0.1–1.6; Table 4). One case presented a double infection, with both HPV16 and HPV62. Among the subgroup of 196 tumour DNA samples that were analysed for the E7 gene from HPV16, all three cases positive for HPV16 by PGMY09/11 PCR were detected. Three tonsillar tumours were additionally positive with the type-specific PCR, resulting in a prevalence of 3.1% (95% CI 1.2–6.3) for HNSCC and 4.4% (95% CI 1.1–11.5) for oropharyngeal samples. With both PCR protocols, we found similar DNA prevalence in oropharyngeal and hypopharyngeal/laryngeal cancer cases. Finally, only 2 out of 1058 controls (0.2%) and 1 out of 1225 cases (0.1%) presented oral cells specimens positive for HPV DNA according to the PGMY09/11 PCR (Table 4). All three PGMY09/11 DNA-positive samples were HPV16 E6 seropositive, and the same was true for two of the three DNA-positive samples additionally detected by the type-specific PCR.

Among HNSCC cases, seropositivity for HPV16 E6 did not differ according to T category or staging group (Table 5). However, HPV16 E6 seroprevalence was significantly higher in HNSCC cases with lymph node involvement (N1–N3, 3.3%), compared with N0 cases (1.8%; OR = 1.99, 95% CI 1.07–3.69). For oropharyngeal cancer, HPV16 E6 seropositive cases presented a 5-fold risk of lymph node metastasis compared with seronegative cases (OR = 5.25, 95% CI 0.96–28.7).

Discussion

Tobacco smoking and alcohol drinking are the main risk factors for HNSCC in most populations.[39] However, recent studies have reported an increased incidence of oral and oropharyngeal cancer, particularly for tongue and tonsillar tumours, among young non-drinkers/non-smokers.[40–44]

In our study, 7.8, 2.5 and 3.2% of the cases have shown a serological response to HPV16L1, HPV16E6 and HPV16E7, respectively, and these data are similar to findings described by Herrero *et al.*,[12] who estimated seroprevalences of 9.6, 3.7 and 3.4%, respectively. However, our study has detected lower seroprevalence of HPV16L1 when compared with other previous studies,[24–27] where seropositivity ranged between 19.4% and 40.8%,[24–27] but it is important to emphasize that these very high seroprevalences can be attributed to samples restricted to never-smokers[24] or with a predominance of oropharyngeal tumours.[25] Furthermore, Zumbach *et al.*[29] have reported slightly higher seropositivity rates for both HPV16E6 (10.9%) and HPV16E7 (6.5%), whereas

Table 3 Risk associated with HPV16E6 serology status for HNSCC and according to tumour site, stratified by age, sex, smoking and alcohol drinking

Variable category	All HNSCC cases				Oral cavity			Oropharynx			Hypopharynx/larynx		
	Cases (±)	Controls (±)	OR[a] (95% CI)	*P*-value*	Cases (±)	OR[a] (95% CI)	*P*-value*	Cases (±)	OR[a] (95% CI)	*P*-value*	Cases (±)	OR[a] (95% CI)	*P*-value*
Age (years)													
⩽50	19/504	5/729	**11.9**[3] **(3.84–36.8)**	**0.020**	2/202	1.47 (0.22–10.0)	0.784	13/97	**62.2**[3] **(12.6–306)**	**0.017**	4/205	**6.14**[1] **(1.10–34.1)**	0.356
>50	37/1654	24/2561	**2.56**[2] **(1.35–4.85)**		9/474	1.98 (0.76–5.12)		11/318	**6.20**[3] **(2.21–17.4)**		17/862	**2.53**[1] **(1.16–5.49)**	
Sex													
Male	36/1887	16/2452	**4.25**[3] **(2.11–8.57)**	0.538	7/570	2.47 (0.84–7.21)	0.369	13/361	**10.8**[3] **(4.02–29.2)**	0.585	16/956	**3.29**[2] **(1.43–7.58)**	0.433
Female	20/271	13/838	**2.98**[1] **(1.23–7.23)**		4/106	1.11 (0.28–4.39)		11/54	**17.7**[3] **(4.14–75.8)**		5/111	1.76 (0.47–6.60)	
Smoking													
Never	10/111	14/1085	**5.28**[2] **(2.11–13.2)**	0.828	1/59	0.91 (0.10–7.81)	0.354	6/9	**45.8**[3] **(11.2–187)**	**0.022**	3/43	**4.55**[1] **(1.13–18.4)**	0.559
Former/current	46/2047	15/2205	**3.32**[3] **(1.76–6.26)**		10/617	**2.79**[1] **(1.10–7.07)**		18/406	**6.58**[3] **(2.75–15.8)**		18/1024	**2.84**[2] **(1.35–5.97)**	
Alcohol drinking													
Never	4/150	6/544	1.54 (0.38–6.29)	0.165	0/51	–	–	2/10	**31.8**[3] **(3.17–319)**	0.418	2/89	0.87 (0.13–5.65)	0.188
Former/current	52/2008	23/2746	**4.54**[3] **(2.48–8.30)**		11/625	2.38 (0.98–5.78)		22/405	**11.5**[3] **(4.95–26.8)**		19/978	**3.41**[2] **(1.59–7.29)**	
Number of sexual partners[b]													
0–10	26/792	7/697	**3.86**[2] **(1.48–10.1)**	**0.050**	7/280	2.18 (0.63–7.62)	0.829	11/159	**16.6**[3] **(3.85–71.3)**	0.126	8/353	2.31 (0.67–8.02)	0.071
⩾11	19/759	1/542	**40.7**[3] **(4.73–351)**		1/222	1.55 (0.09–25.6)		10/156	**167**[3] **(12.8–2188)**		8/381	**26.1**[2] **(2.57–266)**	
Orogenital contact[b]													
Never	28/1210	5/929	**5.82**[3] **(1.97–17.2)**	0.485	7/393	3.26 (0.87–12.3)	0.294	9/249	**20.6**[3] **(4.08–105)**	0.308	12/568	**6.36**[2] **(1.75–23.2)**	0.488
Ever	17/375	3/335	**10.8**[3] **(2.79–41.9)**		1/118	0.72 (0.06–8.69)		12/78	**76.5**[3] **(11.1–527)**		4/179	2.97 (0.53–16.5)	

[a]Odds ratios adjusted by age, sex, smoking, alcohol drinking and country, where appropriate.
[b]The analyses for number of sexual partners and oral genital contact were only performed for LA.
**P*-value for heterogeneity test.
[1]$p < 0.05$; [2]$p < 0.01$; [3]$p < 0.001$.

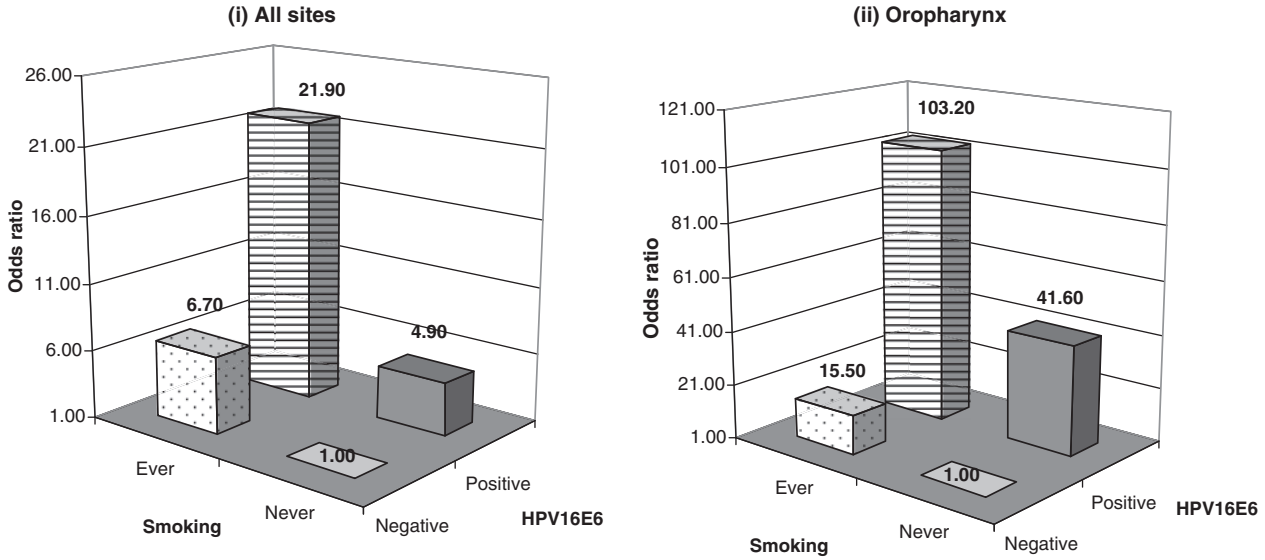

Figure 1 Interaction between HPV16E6 serology status and smoking, for all sites combined and for oropharynx. *P*-value for departure from the multiplicative interaction: (i) $P = 0.47$, (ii) $P = 0.02$

Table 4 HPV16 DNA prevalence in Latin America according to detection method and sample type for HNSCC and individual tumour sites

		Number of specimens analysed; number of positive specimens (%)				
Method	**Sample**	All HNSCC cases	Oral cavity	Oropharynx	Hypopharynx/ larynx	Controls
PGMY09/11	Oral cells	1054; 1 (0.1)	351; 0 (0.0)	218; 0 (0.0)	485; 1 (0.2)	898; 2 (0.2)
PGMY09/11	Frozen tumour	507; 3 (0.6)	132; 0 (0.0)	136; 1 (0.7)	239; 2 (0.8)	NA
HPV16 type-specific PCR	Frozen tumour	196; 6 (3.1)	50; 0 (0.0)	68; 3 (4.4)	78; 3 (3.8)	NA

NA = not applicable.

Smith *et al.*[27] have reported 20.6% and 18.6% for HPV16E6 and HPV16E7, respectively. A recent article from the Czech Republic[45] has reported seropositivity rates of 48.8% for HPV16 E6 and 38.4% for HPV16 E7,but it is important to highlight that the study described a population where 23.3% and 3.5% of the cases were never-drinkers and never-smokers, respectively, whereas in comparison, our corresponding figures for never-drinkers and never-smokers in CE were 1.5 and 6.1%, respectively. In addition, this variation in the seroprevalence across studies may be explained by the proportion of specific HNSCC topographies or differences in the characteristics of the assays.[27] In our study, only 19.8% of all HNSCC were oropharyngeal cancers as compared with 30.4% and 37.5% in the previously cited studies, respectively.[27,29] However, even when restricting the comparison to the oropharyngeal cancers, antibodies against HPV16 E6 and/or E7 were detected in only 7.7% of our cases, compared with the very high seropositivity rates (64%) reported in other studies.[8,27] In contrast, the observed seropositivity rate for HPV16E6 and/or E7 in our study is very similar to that reported in the only other study of comparable size, either for HNSCC overall (4.7% in our study vs 4.6%)[12] or for oropharyngeal cancer (7.7% in our study compared with 11.9% reported by Herrero *et al.*[12]).

Our algorithm for defining cut-off levels was developed a priori and was designed to minimize false positive results. Given that HPV16 E6 and E7 oncoproteins are generally viewed as markers of invasive HPV-related tumours, our background control levels of ~1–2% are probably reasonable. Lowering cut-off levels generally led to an increased prevalence of E6 and E7 antibodies in controls, although an attenuation in any case–control difference. For example, sensitivity analysis using a reduced cut-off level of 100 MFI for HPV16 E6 and E7 resulted in a background prevalence in controls of 3.4% instead of 0.9% for E6 and 15% instead of 1.7% for E7. The low HPV seropositivity observed in the study population is also corroborated by the HPV DNA data. Only 3

Table 5 Association between HPV16 E6 serology status and staging variables for HNSCC and according to tumour site

Variable	All HNSCC cases ($n = 1949$) (±)	OR[a] (95%CI)	Oral cavity		Oropharynx		Hypopharynx/larynx	
			±	OR[a] (95%CI)	±	OR[a] (95%CI)	±	OR[a] (95%CI)
T stage								
T1s–T2	22/713		3/215	1.0	8/97	1.0	11/401	1.0
T3–T4	28/1205	0.80 (0.45–1.45)	8/400	1.45 (0.37–5.74)	11/259	0.59 (0.19–1.80)	9/546	0.62 (0.25–1.54)
N stage								
N0	18/1012		4/284	1.0	2/139	1.0	12/589	1.0
N1, N2, N3	30/889	**1.99 (1.07–3.69)**[1]	7/308	1.66 (0.46–6.06)	15/209	5.25 (0.96–28.7)	8/372	1.12 (0.44–2.87)
Staging group								
0–II	9/516		2/159	1.0	1/52	1.0	6/305	1.0
III–IV	40/1384	1.11 (0.43–2.89)	9/441	2.04 (0.23–18.3)	17/299	0.45 (0.02–9.28)	14/644	0.98 (0.30–3.15)

[a]OR adjusted by age, sex, country, smoking and alcohol drinking.
[1]$p < 0.05$.

tumours in 507 were found to harbour HPV DNA. This low prevalence could be attributed to the under-representation of tumour cells in the specimens analysed, since microdissection was not performed. Such an explanation is, however, unlikely given that p53 mutations were detected in >50% of samples,[46] confirming that our samples are largely made up of tumour cells. In addition, the PCR method employed in our study (PGMY09/11) is the most frequently used in HPV DNA detection by PCR, due to its wide genotype coverage and adequate sensitivity.

PAR for HPV16 E6, E7 and either/both E6/E7 seropositivity and HNSCC were 2.6, 2.8 and 2.2%, respectively, whereas for oropharyngeal cancer, corresponding figures were 8.8, 8.1 and 5.3% (Table 6). These estimates are similar to those obtained for the IARC Multicenter Study (8.8, 8.1 and 7.7%, respectively),[12] but still very small when compared with data described by Smith *et al*.[27] and D'Souza *et al*.,[8] that reported a PAR of 53.0% and 62.5% for all oropharyngeal cancer cases being attributed to HPV16 E6/E7 (either or both positive). In addition, the percentage of never-smokers or never-drinkers among subjects with oropharyngeal cancer varied across different studies: 3.4% and 2.7% in our series, 9% and 14.9% in the IARC Multicenter Study[12] and 44% and 15% reported by D'Souza *et al*.[8], respectively. One interpretation of this finding is that the proportion of head and neck cancers associated with HPV can be smaller in countries where the burden of the classical risk factors—tobacco and alcohol—is high.[12]

Contrary to other studies,[8,24–26,28] we could not find a significant association between serological response to HPV16L1 and HNSCC, but it is important to emphasize that this association is always low.[12] Antibodies against HPV16 L1 are considered markers of cumulative exposure to the virus, but not all exposed individuals present seroconversion and/or maintain detectable levels over time.[22] In addition, Kreimer *et al*.[47] reported that oral and oropharyngeal squamous cell carcinomas with a low viral load have lower prevalence of seropositivity for HPV16 L1 (22.2%) when compared with cases with a high viral load (60.0% seropositive). Furthermore, it has been demonstrated that HPV16 virus-like particle (VLP) seropositivity alone presents lower concordance with HPV DNA in tumour tissues than antibodies to E6 or E7.[48] The sensitivity of E6 and E7 antibodies, however, is also incomplete, and likely to be ~50%.[49] This indicates that we may have underestimated our PAR by a similar amount, although they would remain modest even after such a correction.

Our results show a strong association between antibodies to the HPV16 oncoproteins E6/E7 and HNSCC. Serological response to HPV oncoproteins E6 and E7 appears to be a specific marker for HPV-associated cancers.[50,51] It is still not well known why the seroreactivity to proteins E6 and E7 is more frequent after tumour invasion, but the major hypothesis is that there is development of a tumour vascular bed, followed by the release of E6 and E7 after necrosis and spread to the systemic circulation, generating a humoral response.[51,52]

The antibody pattern we observed in our study, with an important percentage of double positives for both HPV16 oncoproteins, is similar to findings reported by other authors for head and neck[29] and cervical cancer.[50,51] One could argue that HPV 16 infects primarily anogenital sites and, since a serological test is not site-specific, cancers outside the head and neck region might be responsible for the observed associations. Nevertheless, it is improbable that this association is biased due to non-diagnosed HPV-related cancers in other anatomical sites, since we have excluded cases and controls with other cancer types. Furthermore, it is important to emphasize that only three controls (0.1%) were positive for both HPV16E6 and HPV16E7.

Table 6 Comparison of prevalence (P) and population-attributable fractions (PAF) for antibodies to HPV16 E6, HPV16 E7, and HPV16 E6 and/or E7 and HNSCC or oropharyngeal cancer, across different studies

Study	Region	Cases	Controls	HPV16 E6				HPV16 E7				HPV16 E6&E7 (either or both)			
				HNSCC		Oropharynx		HNSCC		Oropharynx		HNSCC		Oropharynx	
				P	PAF	P	PAF	P	PAF	P	PAF	P	PAF	P	PAF
Current study—LA	Argentina, Brazil, Cuba	1670	1288	2.7	2.1	6.1	5.5	3.6	1.6	6.9	4.9	5.1	2.6	8.9	6.4
Current study—CE	Czech Republic, Poland, Romania, Russia, Slovakia	544	2031	1.8	0.8	2.5	1.5	1.6	0.1	1.3	0.3	3.3	0.9	2.5	0.1
Zumbach *et al.*[29]	Germany	92	288	10.9	9.3	–	–	7.6	6.6	–	–	11.9	9.4	–	–
Herrero *et al.*[12]	Australia, Canada, Cuba, India, Italy, Northern Ireland, Poland, Spain, Sudan	1562	1581	3.7	2.6	9.9	8.8	3.5	2.8	8.6	8.1	5.8	1.2	11.9	7.7
Smith *et al.*[27]	USA	204	326	20.6	19.8	53.2	52.8	18.6	18.1	53.2	52.9	24.5	23.6	64.5	53.0
D'Souza *et al.*[8]	USA	100	200	–	–	–	–	–	–	–	–	–	–	64.0	62.5
Tachezy *et al.*[45]	Czech Republic	86	104	48.8	48.3	56.0	55.6	38.4	36.5	44.0	42.3	48.8	46.8	56.0	54.2

Seropositivity for both HPV16 E6 and E7 was also a statistically significant risk factor for hypopharyngeal/laryngeal cancer in our analysis. Data from other studies have already pointed out that HPV is possibly also involved in the pathogenesis of a small proportion of laryngeal carcinomas.[28,53] Applebaum *et al.*[28] have reported that HPV16 L1 seropositivity was associated with an increased risk of laryngeal cancer (OR = 2.7, 95% CI 1.5–5.1). Moreover, it is well described that HPV, mainly types 6 and 11, is the causal agent of recurrent respiratory papillomatosis (RPP)[54] with a prevalence of up to 100%.[55] In a small percentage of cases, laryngeal papillomatosis may undergo malignant transformation into laryngeal squamous cell carcinoma.[54] Therefore, there is evidence to support the association between HPV and a subgroup of laryngeal cancers, but it is still necessary to investigate the role of cofactors in this process.[54] A recent evaluation of HPV carcinogenicity within the IARC Monographs programme has confirmed the classification of HPV16 as carcinogenic to humans (Group 1), based on a causal association with genital cancers, oral cavity and oropharyngeal cancers, whereas for laryngeal cancer, evidence was still considered limited.[56] Results from our study provide additional evidence for an association between hypopharyngeal/laryngeal cancer for both HPV16 E6 and joint detection of E6/E7.

The association between HPV16 E6 seropositivity and oropharyngeal cancer was stronger for young individuals ($\leqslant 50$ years), which is consistent with some previous studies.[27,48,57] The high prevalence of HPV-positive tumours among young individuals may be explained by high-risk sexual behaviours such as number of partners, orogenital and oral–anal sex, as previously demonstrated.[57] Furthermore, our finding of an interaction between HPV16 seropositivity and the number of sexual partners is in agreement with previous reports.[23,53] Recently, D'Souza *et al.*[58] have demonstrated that both number of lifetime vaginal or oral sex partners were independently associated with prevalent oral HPV infection.

Interactions among HPV, tobacco smoking and alcohol drinking have been evaluated in several studies and results are inconsistent.[12,23] We were able to find an interaction between smoking and HPV16E6 seropositivity only for oropharyngeal cancer, with a stronger association among never-smokers compared with ever/former smokers, as already described in previous studies.[8,12,28] One possible mechanism to explain this interaction is that, after the inactivation of p53 and pRb by HPV proteins E6 and E7, respectively, the additional clonal selection associated with tobacco smoking and alcohol drinking will operate on other pathways, which are different from p53 and pRb.[28]

The serological response to a non-HPV16 high-risk type, HPV52, was associated with a 9-fold risk of oropharyngeal cancer after exclusion of potentially

confounding HPV16-seropositive subjects. However, DNA information (by PGMY09/11 PCR on tumour tissue) was available for three of the six HPV52 E6 seropositive cases, and no HPV52 DNA had been found in these three samples. HPV52 is classified as oncogenic and belongs together with the HPV types 16, 31, 33, 35 and 58 to the alpha 9 species of the HPV phylogenetic tree, characterized by similar pathogenic properties.[59] The worldwide prevalence of HPV52 in cervical cancer patients is 2.7%, but in South America it is the fifth most common HPV type among women with cervical cancer, with a prevalence of 4.2%.[60] This is the first study to show this association and it is possible that, in areas with a high prevalence of HPV 52, this can have a role in the development of oropharyngeal cancer, but additional evidence is still required.

HNSCC cases that were seropositive for HPV16 E6 had a higher risk of lymph node metastasis compared with seronegative cases. It is possible that such an association may be due to heightened serological response in the presence of an increased tumour burden. The association with HPV16 E6 was restricted to oropharyngeal cancer cases. This finding is in agreement with other studies[27,48] and this association can be supported by the results reported by Pyeon *et al.*,[61] demonstrating that a subgroup of HPV DNA-positive head and neck cancers has a genetic profile characterized by overexpression of the cell-migration regulator synaptopodin which, in turn, has been associated with increased invasiveness. Nevertheless, no association was found between seropositivity for HPV16 (E6; E6 and/or E7) and tumour size (TNM T category) or stage group.

The results of our study provide further evidence on the aetiological link between HPV and oropharyngeal cancer. Furthermore, we present new data on the association between laryngeal cancer and HPV. However, in these two high-risk populations characterized by high prevalence of tobacco smoking and alcohol drinking, the overall HPV prevalence in HNSCC was substantially lower than that described in US studies. This suggests that the impact of HPV vaccination on HNSCC may be not significant in these populations and that prevention of these cancers should remain focussed on programmes for reducing alcohol and tobacco consumption.

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Conflict of interest: None declared.

KEY MESSAGES

- Recent studies of HPV serology and cancer of the head and neck from the US report a prevalence of HPV 16 E6 or E7 antibodies of ~20% among all cases, and >50% for cases of the oropharynx.
- Incidence of head and neck cancers is high in countries of Latin America and Central Europe. In our series of >2000 cases and 3000 controls, we report a very low prevalence of HPV 16 E6 or E7 antibodies (<5%) in both head and neck cancers overall and in cancers of the oropharynx. These results were supported by analysis of tumour DNA.
- The proportion of head and neck cancers caused by HPV does not appear to be high in these regions.

References

[1] Ferlay J, Bray F, Pisani P, Parkin DM. *GLOBOCAN 2002*. Lyon: IARC Press, 2004.

[2] Andl T, Kahn T, Pfuhl A *et al*. Etiological involvement of oncogenic human papillomavirus in tonsillar squamous cell carcinomas lacking retinoblastoma cell cycle control. *Cancer Res* 1998;**58:**5–13.

[3] Snijders PJ, Steenbergen RD, Top B, Scott SD, Meijer CJ, Walboomers JM. Analysis of p53 status in tonsillar carcinomas associated with human papillomavirus. *J Gen Virol* 1994;**75**(Pt 10):2769–75.

[4] Snijders PJ, Scholes AG, Hart CA *et al*. Prevalence of mucosotropic human papillomaviruses in squamous-cell carcinoma of the head and neck. *Int J Cancer* 1996;**66:** 464–69.

[5] Snijders PJ, Steenbergen RD, Meijer CJ, Walboomers JM. Role of human papillomaviruses in cancer of the respiratory and upper digestive tract. *Clin Dermatol* 1997;**15:** 415–25.

[6] van Houten VM, Snijders PJ, van den Brekel MW *et al*. Biological evidence that human papillomaviruses are etiologically involved in a subgroup of head and neck squamous cell carcinomas. *Int J Cancer* 2001;**93:**232–35.

[7] Wiest T, Schwarz E, Enders C, Flechtenmacher C, Bosch FX. Involvement of intact HPV16 E6/E7 gene expression in head and neck cancers with unaltered p53 status and perturbed pRb cell cycle control. *Oncogene* 2002;**21:**1510–17.

[8] D'Souza G, Kreimer AR, Viscidi R *et al*. Case-control study of human papillomavirus and oropharyngeal cancer. *N Engl J Med* 2007;**356:**1944–56.

[9] Gillison ML, Shah KV. Human papillomavirus-associated head and neck squamous cell carcinoma: mounting evidence for an etiologic role for human papillomavirus in a subset of head and neck cancers. *Curr Opin Oncol* 2001;**13:** 183–88.

[10] Gillison ML. Human papillomavirus-associated head and neck cancer is a distinct epidemiologic, clinical, and molecular entity. *Semin Oncol* 2004;**31:**744–54.

[11] Habeck M. New data confirm that HPV can cause oropharyngeal cancer. *Mol Med Today* 2000;**6:**297.

[12] Herrero R, Castellsague X, Pawlita M *et al*. Human Papillomavirus and Oral Cancer: The International Agency for Research on Cancer Multicenter Study. *J Natl Cancer Inst* 2003;**95:**1772–83.

[13] Hobbs CG, Sterne JA, Bailey M, Heyderman RS, Birchall MA, Thomas SJ. Human papillomavirus and head and neck cancer: a systematic review and meta-analysis. *Clin Otolaryngol* 2006;**31:**259–66.

[14] Smith EM, Hoffman HT, Summersgill KS, Kirchner HL, Turek LP, Haugen TH. Human papillomavirus and risk of oral cancer. *Laryngoscope* 1998;**108:**1098–103.

[15] Smith EM, Summersgill KF, Allen J *et al*. Human papillomavirus and risk of laryngeal cancer. *Ann Otol Rhinol Laryngol* 2000;**109:**1069–76.

[16] Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiol Biomarkers Prev* 2005;**14:**467–75.

[17] Paavonen J, Jenkins D, Bosch FX *et al*. Efficacy of a prophylactic adjuvanted bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a phase III double-blind, randomised controlled trial. *Lancet* 2007;**369:**2161–70.

[18] Villa LL, Costa RL, Petta CA *et al*. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. *Lancet Oncol* 2005;**6:**271–78.

[19] Gillison ML, Chaturvedi AK, Lowy DR. HPV prophylactic vaccines and the potential prevention of noncervical cancers in both men and women. *Cancer* 2008; **113(Suppl 10):**3036–46.

[20] Aburad ATT. Expression of cyclin D1, c-jun and retinoblastoma protein and HPV detection in oral squamous cell carcinomas. PhD Thesis. School of Dentistry, University of São Paulo 2006.

[21] Rivero ER, Nunes FD. HPV in oral squamous cell carcinomas of a Brazilian population: amplification by PCR. *Braz Oral Res* 2006;**20:**21–24.

[22] Dillner J. The serological response to papillomaviruses. *Semin Cancer Biol* 1999;**9:**423–30.

[23] Schwartz SM, Daling JR, Doody DR *et al*. Oral cancer risk in relation to sexual history and evidence of human papillomavirus infection. *J Natl Cancer Inst* 1998;**90:** 1626–36.

[24] Dahlstrom KR, dler-Storthz K, Etzel CJ *et al*. Human papillomavirus type 16 infection and squamous cell carcinoma of the head and neck in never-smokers: a matched pair analysis. *Clin Cancer Res* 2003;**9:**2620–26.

[25] Furniss CS, McClean MD, Smith JF *et al*. Human papillomavirus 16 and head and neck squamous cell carcinoma. *Int J Cancer* 2007;**120:**2386–92.

[26] Pintos J, Black MJ, Sadeghi N *et al*. Human papillomavirus infection and oral cancer: A case-control study in Montreal, Canada. *Oral Oncol* 2008;**44:**242–50.

[27] Smith EM, Ritchie JM, Pawlita M *et al*. Human papillomavirus seropositivity and risks of head and neck cancer. *Int J Cancer* 2007;**120:**825–32.

[28] Applebaum KM, Furniss CS, Zeka A *et al*. Lack of association of alcohol and tobacco with HPV16-associated head and neck cancer. *J Natl Cancer Inst* 2007;**99:**1801–10.

[29] Zumbach K, Hoffmann M, Kahn T *et al*. Antibodies against oncoproteins E6 and E7 of human papillomavirus types 16 and 18 in patients with head-and-neck squamous-cell carcinoma. *Int J Cancer* 2000;**85:**815–18.

[30] Guha N, Boffetta P, Wunsch F *et al*. Oral health and risk of squamous cell carcinoma of the head and neck and esophagus: results of two multicentric case-control studies. *Am J Epidemiol* 2007;**166:**1159–73.

[31] Sehr P, Zumbach K, Pawlita M. A generic capture ELISA for recombinant proteins fused to glutathione S-transferase: validation for HPV serology. *J Immunol Methods* 2001;**253:**153–62.

[32] Sehr P, Muller M, Hopfl R, Widschwendter A, Pawlita M. HPV antibody detection by ELISA with capsid protein L1 fused to glutathione S-transferase. *J Virol Methods* 2002; **106:**61–70.

[33] Waterboer T, Sehr P, Michael KM *et al*. Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins. *Clin Chem* 2005;**51:** 1845–53.

[34] Waterboer T, Sehr P, Pawlita M. Suppression of non-specific binding in serological Luminex assays. *J Immunol Methods* 2006;**309:**200–04.

[35] Clifford GM, Shin HR, Oh JK *et al*. Serologic response to oncogenic human papillomavirus types in male and female university students in Busan, South Korea. *Cancer Epidemiol Biomarkers Prev* 2007;**16:**1874–79.

[36] Michael KM, Waterboer T, Sehr P *et al*. Seroprevalence of 34 human papillomavirus types in the German general population. *PLoS Pathog* 2008;**4:**e1000091.

[37] Coutlee F, Gravitt P, Kornegay J *et al*. Use of PGMY primers in L1 consensus PCR improves detection of human papillomavirus DNA in genital samples. *J Clin Microbiol* 2002;**40:**902–07.

[38] Bernard HU, Chan SY, Manos MM *et al*. Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphisms, nucleotide sequence, and phylogenetic algorithms. *J Infect Dis* 1994; **170:**1077–85.

[39] Curado MP, Hashibe M. Recent changes in the epidemiology of head and neck cancer. *Curr Opin Oncol* 2009;**21:** 194–200.

[40] Golas SM. Trends in palatine tonsillar cancer incidence and mortality rates in the United States. *Community Dent Oral Epidemiol* 2007;**35:**98–108.

[41] Chaturvedi AK, Engels EA, Anderson WF, Gillison ML. Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. *J Clin Oncol* 2008;**26:**612–19.

[42] Kolker JL, Ismail AI, Sohn W, Ramaswami N. Trends in the incidence, mortality, and survival rates of oral and pharyngeal cancer in a high-risk area in Michigan, USA. *Community Dent Oral Epidemiol* 2007;**35:**489–99.

[43] Shiboski CH, Schmidt BL, Jordan RC. Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20–44 years. *Cancer* 2005;**103:**1843–49.

[44] Annertz K, Anderson H, Biorklund A *et al*. Incidence and survival of squamous cell carcinoma of the tongue in Scandinavia, with special reference to young adults. *Int J Cancer* 2002;**101:**95–99.

[45] Tachezy R, Klozar J, Rubenstein L *et al*. Demographic and risk factors in patients with head and neck tumors. *J Med Virol* 2009;**81:**878–87.

[46] Szymanska K, Levi JE, Menezes A *et al*. TP53 and EGFR mutations in combination with lifestyle risk factors in tumours of the upper aerodigestive tract from South America. *Carcinogenesis* 2010;**31:**1054–59.

[47] Kreimer AR, Clifford GM, Snijders PJ *et al*. HPV16 semiquantitative viral load and serologic biomarkers in oral and oropharyngeal squamous cell carcinomas. *Int J Cancer* 2005;**115:**329–32.

[48] Smith EM, Rubenstein LM, Ritchie JM *et al*. Does pretreatment seropositivity to human papillomavirus have prognostic significance for head and neck cancers? *Cancer Epidemiol Biomarkers Prev* 2008;**17:** 2087–96.

[49] Meschede W, Zumbach K, Braspenning J *et al*. Antibodies against early proteins of human papillomaviruses as diagnostic markers for invasive cervical cancer. *J Clin Microbiol* 1998;**36:**475–80.

[50] Zumbach K, Kisseljov F, Sacharova O *et al*. Antibodies against oncoproteins E6 and E7 of human papillomavirus types 16 and 18 in cervical-carcinoma patients from Russia. *Int J Cancer* 2000;**85:**313–18.

[51] Meschede W, Zumbach K, Braspenning J *et al*. Antibodies against early proteins of human papillomaviruses as diagnostic markers for invasive cervical cancer. *J Clin Microbiol* 1998;**36:**475–80.

[52] Stanley M. Antibody reactivity to HPV E6 and E7 oncoproteins and early diagnosis of invasive cervical cancer. *Am J Obstet Gynecol* 2003;**188:**3–4.

[53] Gillison ML, D'Souza G, Westra W *et al*. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. *J Natl Cancer Inst* 2008;**100:**407–20.

[54] Torrente MC, Ojeda JM. Exploring the relation between human papilloma virus and larynx cancer. *Acta Otolaryngol* 2007;**127:**900–06.

[55] Levi JE, Delcelo R, Alberti VN, Torloni H, Villa LL. Human papillomavirus DNA in respiratory papillomatosis detected by in situ hybridization and the polymerase chain reaction. *Am J Pathol* 1989;**135:**1179–84.

[56] Bouvard V, Baan R, Straif K *et al*. A review of human carcinogens—Part B: biological agents. *Lancet Oncol* 2009;**10:**321–22.

[57] Smith EM, Ritchie JM, Summersgill KF *et al*. Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers. *Int J Cancer* 2004;**108:** 766–72.

[58] D'Souza G, Agrawal Y, Halpern J, Bodison S, Gillison ML. Oral sexual behaviors associated with prevalent oral human papillomavirus infection. *J Infect Dis* 2009;**199:** 1263–69.

[59] Huang S, Afonina I, Miller BA, Beckmann AM. Human papillomavirus types 52 and 58 are prevalent in cervical cancers from Chinese women. *Int J Cancer* 1997;**70:** 408–11.

[60] Castellsague X, de Sanjosé S, Aguado T *et al*. HPV and cervical cancer in the world: 2007 report. *Vaccine* 2007; **25(Suppl 3):**C1–C230.

[61] Pyeon D, Newton MA, Lambert PF *et al*. Fundamental differences in cell cycle deregulation in human papillomavirus-positive and human papillomavirus-negative head/neck and cervical cancers. *Cancer Res* 2007;**67:**4605–19.